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(54) Title: ENHANCEMENT OF CELL GROWTH BY EXPRESSION OF CLONED OXYGEN-BINDING PROTEINS

(57) Abstract

Methods for the use of DNA encoding oxygen-binding proteins and related plasmids containing same are disclosed for a range of applications including oxygen supply to cells, growth enhancement, expression of various gene products, enhancement of oxygen-requiring processes, binding and separation of oxygen from liquids and gases, and a range of oxidative reactions.

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ENHANCEMENT OF CELL GROWTH BY EXPRESSION OF CLONED OXYGEN-BINDING PROTEINS

The United States Government has rights in this invention pursuant to Grant No. BCS-8805636 from the National Science Foundation.

This is a continuation-in-part of Ser. No. 07/887169, filed May 20, 1992, which is a continuation-in-part of Serial No. 07/741,789, filed August 6, 1991, which is a continuation of Serial No. 342,451, filed October 21, 1988, now U.S. 5,049,493, which is a continuation-in-part of Serial No. 151,256, filed February 28, 1988, abandoned, and of Serial No. 113,014, filed October 23, 1987, abandoned, all of which are incorporated by reference herein.

TECHNICAL FIELD

This invention relates to the production of oxygen-binding proteins, particularly members of the globin family, and to enhancement of the growth and product synthesis characteristics of aerobic organisms in environments with sufficient as well as reduced or low levels of oxygen.

This invention relates generally to the use of recombinant DNA technology to direct or otherwise control gene expression in cultured cells, and more particularly, to methods and materials useful in subjecting the transcription and translation of DNA

sequences to selective r gulation by external control.

BACKGROUND ART

The present invention is directed toward the expression and use of oxygen-binding proteins, including, but not limited to, the evolutionary-related globin superfamily: hemoglobins (found in many eukaryotic organisms); leghemoglobins (found in many plants); myoglobins (found mainly in animal muscle tissue); and microbial globins such as Vitreoscilla hemoglobin; E. coli hmp (a flavohemoglobin), and yeast flavohemoglobin. Other oxygen-binding hemoproteins include cytochrome C reductase, cytochrome c oxidase, and kinase in the oxygen sensor of Rhizobium meliloti. Additional oxygen-binding proteins which function in the transport of oxygen in invertebrates include hemocyanins and hemerythrins. The existence of Vitreoscilla hemoglobin, E. coli hmp (Mol. Gen. Genet. 226: 49-58, 1991) and yeast flaxohemoglobin (F.A.S.E.B. J. 6: A318, 1992), incorporated herein by reference, suggests that globin-like oxygen-binding proteins may also be distributed in microrganisms. Such endogenous, homologous globins may also be utilized in accordance with the present invention, providing the added advantage of expressing a globin from a gene homologous to the desired host organism. By reversibly binding to oxygen in the presence of high oxygen concentrations and releasing it in regions or at times of low concentrations, these oxygen-binding proteins can considerably enhance the oxygen uptake rate of multicellular organism over that allowed by mere passive diffusion. In unicellular organisms it is generally believed that the oxygen uptake rate is principally limited by

the rate of transfer of dissolved oxygen in the environment or growth medium to the exterior cell surface. However, closer examination of cell structure reveals several potential diffusional barriers between environmental oxygen and the cytochromes where the oxygen finally undergoes reaction. For example, in gram negative bacteria, where the cytochromes are attached to the inside of the plasma membrane, the diffusing oxygen needs to cross transport barriers such as the cell wall, the outer membrane, the periplasmic space and the inner membrane before accepting electrons from metabolic reactions. In unicellular eucaryotes, where oxidative phosphorylation takes place in the mitochondria, there are further diffusional resistances. Small neutral molecules like oxygen are assumed to passively diffuse across these barriers; however, these barriers make a non-trivial contribution to the overall resistance to mass transfer to the actual reaction site and thus could be of significance under conditions of low oxygen or oxygen limitation.

Physiological effects on growth due to depletion in dissolved oxygen levels has been demonstrated in the case of several organisms, including Escherichia coli, Saccharomyces cerevisiae, Pseudomonas strains, and Alcaligenes eutrophus. In E. coli for example, which has a very high affinity cytochrome, changes in dissolved oxygen tension leads to differential regulation of terminal oxidases, resulting in a decrease in the number of protons expelled per NADH molecule oxidized during aerobic respiration and, consequently, a possible adverse change in the stoichiometry of ATP biosynthesis. (Kranz et al., Journal of Bacteriology 158:1191-1194, 1984; Ingraham et al., Growth of the bacterial cell, Sinauer

Associates, Inc. 1983, p. 147, both specifically incorporated herein.)

In addition to the respiratory oxygen requirement of aerobic organisms, oxygen-binding proteins have other potential applications as well, including, for example, the enhancement of particular oxidative transformations such as steroid conversions, vinegar production, biological waste treatment or enzymatic degradations, and in some steps in brewing or making distilled and fermented foods and beverages.

The filamentous bacterium, Vitreoscilla, a member of the Beggiatoa family, is a strict aerobe that is found in oxygen-poor environments such as stagnant ponds and decaying vegetable matter. Growth of the bacterium under hypoxic conditions results in a several-fold induction of synthesis of a homodimeric soluble heme protein (subunit MW 15,775) (Boerman et al., Control of heme content in Vitreoscilla by oxygen, Journal of General Applied Microbiology 28:35-42, 1982) which has a remarkable spectral (Webster, et al., Reduced nicotinamide adenine dinucleotide cytochrome o reductase associated with cytochrome o purified from Vitreoscilla, Journal of Biological Chemistry 249:4257-4260, 1974), structural (Wakabayashi, et at., Primary sequences of a dimeric bacterial hemoglobin from Vitreoscilla, Nature 322:481-483, 1986), and kinetic (Orii, et al., Photodissociation of oxygenated cytochrome o(s) (Vitreoscilla) and kinetic studies of reassociation, Journal of Biological Chemistry 261:2978-2986, 1986) homology with eucaryotic hemoglobins, and which is probably a true bacterial hemoglobin.

This protein was previously thought to be a cytochrome o, and it has been suggested to function

in oxygen storage. However, biochemical discrepancies (Webster, et al., Oxygenated cytochrome o, Journal of Biological Chemistry 252:1834-1836, 1977) as well as the subsequent discovery of the true membrane-bound cytochromes o and d (DeMaio, et al., Spectral evidence for the existence of a second cytochrome o in whole cells of Vitreoscilla, Journal of Biological Chemistry 258:13768-13771, 1983; Webster et al., Federation Proceeding 44:678, 1985) led to further investigations of its spectral properties (Choc et al., Oxygenated intermediate and carbonyl species of cytochrome o (Vitreoscilla), Journal of Biological Chemistry 257: 865-869, 1982; Orii et al., supra.) and the eventual determination of its probable amino acid sequences and partial homology with known hemoglobin sequences (23).

Although these articles disclose the conservation of most features characteristic of eukaryotic hemoglobins, and discuss, to some extent, the role or potential role it probably plays in oxygen utilization, it is not believed to have been suggested by others that there is any benefit from the introduction of a bacterial hemoglobin or other oxygen-binding proteins in heterologous organisms. Moreover, it is believed that there has been no suggestion that such oxygen-binding proteins would have a far-reaching range of applications.

Surprisingly, the present inventors have discovered that oxygen-binding proteins are useful in enhancing oxygen supply to cells or in other oxygen-utilizing processes, and for binding and separating oxygen from other fluids or gases. Furthermore, the oxygen-binding proteins are capable of increasing production of cells, or of proteins or metabolites normally made by a cell, or of natural or unnatural metabolites and

proteins expressed in a cell via genetic manipulation. These proteins are also useful as selective markers in recombinant-DNA work, and have applications as diverse as enhancing certain oxygen-requiring steps in fermentation, enzymatic degradation, toxic chemical waste treatment, brewing, and particular oxidative reactions and transformations.

A preferred method of expressing these proteins in bacteria is to use the promoter/regulator sequence of V. hemoglobin of control expression of the homologous oxygen-binding protein. The DNA sequences which usually precede a gene in a DNA polymer and which provide a site for initiation of the transcription of that gene into mRNA. These are referred to as "promoter" sequences. Other DNA or RNA sequences, also usually but not necessarily "upstream" of a structural gene, bind proteins that determine the frequency or rate of transcription and/or translation initiation. These other sequences, including attenuators, enhancers, operators and the like, are referred to as "regulator" sequences. Thus, sequences which operate to determine whether the transcription and eventual expression of a gene will take place are collectively referred to as "promoter/regulator" DNA sequences.

The lactose ("lac") promoter/operator systems have also been commonly used, for they are very controllable through the mode of action of the operator. When the operator is repressed, the DNA dependent RNA polymerase is completely prevented from binding and initiating transcription, thus effectively blocking promoter operability. This system can be derepressed by induction following the addition of a known inducer, such as isopropyl-beta-

D-thiogalactoside (IPTG). The inducer causes the repressor protein to fall away so the RNA polymerase can function.

Cells transformed with plasmids carrying the lac promoter/operator system can be permitted to grow up to maximal density while in the repressed state through the omission of an inducer, such as IPTG, from the media. When a high level of cell density is achieved, the system can be derepressed by addition of inducer. The promoter is then free to initiate transcription and thus obtain expression of the gene products at yields commensurate with the promoter strength. However, certain of these inducible promoter systems are relatively weak and commercial or research productions using such systems do not urge the cell to generate maximum output.

In response to the need for microbial expression vehicles capable of producing desired products in higher yield, the tryptophan ("trp") promoter/operator system has become widely used. This system is one of several known systems with at least three times the strength of the lac promoter. However, it has the disadvantage of less promoter control. The trp promoter is not inducible in the way the lac promoter is, namely, the bound repressor is not removed by induction. Instead, the system operates on a sort of feedback loop as described above. A system was devised whereby the attenuator region of the trp promoter/operator system was removed, with the resultant transformed cells being grown in tryptophan-rich media. This provided sufficient tryptophan to essentially completely repress the operator so that cell growth could proceed uninhibited by premature expression of any desired foreign proteins. When the culture reached

appropriate growth levels, no additional tryptophan was supplied, resulting in mild tryptophan limitation, and, accordingly, derepression of the promoter with resultant expression of th desired protein gene insert. In application, this system has several disadvantages. For example; it is necessary to maintain high levels of tryptophan in the growth media to completely repress the promoter, and to permit the medium to become completely exhausted of tryptophan following full growth of the culture.

A hybrid system has been developed from the tryptophan and lactose promoter, wherein both promoters can be repressed by the lac repressor and both can be derepressed with IPTG. See De Boer et al., The tac promoter: A functional hybrid derived from the trp and lac promoters, Proc. Natl. Acad. Sci. USA, 80: 21-25, 1983. This system shares a disadvantage with the two discussed above, namely the required introduction of additional agents to a normal growth medium.

Another regulator/promoter system commonly used for expression of cloned proteins in E. coli is based on the P_L promoter system from phage lambda. See Bernard and Helsinki, Methods in Enzymology, 68:482-492, 1979; Use of Lambda Phage Promoter P_L to Promote Gene Expression In Hybrid Plasmid Cloning Vehicles. Induction of this promoter requires increase of culture temperature from 30°C to 42°C. This system has the disadvantages of suboptimal growth rates at 30°C prior to induction and upsetting of cell metabolism by the temperature shift. Temperature shift effects on metabolism are discussed, for example, by Neidhart, et. al., The Genetics And Regulation Of Heat-Shock Proteins, Annual Reviews of Genetics, 18:295-329, 1984.

The promoter systems described above are thus useful for expression of proteins of the globin family.

DISCLOSURE OF THE INVENTION

The present invention relates to the expression and use of oxygen-binding proteins, particularly hemoglobins, recombinant-DNA methods of producing same, and to portable DNA sequences capable of directing intracellular production of these oxygen-binding proteins.

The present invention provides novel methods and materials for subjecting DNA sequences of living microorganisms to external regulation which is dependent upon availability of oxygen in the environment. Particularly, it relates to promoter/regulators, a recombinant-DNA method of producing same, and to portable DNA sequences capable of directing the translation and transcription initiation and control of the expression of desired gene products.

Thus, another object of the present invention is provide for the control of expression of any selected chromosomal or extrachromosomal gene or DNA sequence through the incorporation of a promoter/regulator DNA sequence which is functionally responsive to environmental variations in the concentration of oxygen. The invention is thus broadly applicable to a variety of aerobic or slightly aerobic procedures for controlling genetic processes, ranging from the alteration of existing regulation of endogenous genes in prokaryotic and eucaryotic cells to securing selective, differential regulation of expression of selected exogenous or foreign genes stably incorporated in host cells.

Additional objects and advantages of the invention will be set forth in part in the description which foll ws, and in part will be obvious from the description or may be learned from practice of the invention. The objects and advantages may be realized and attained by means of the instrumentalities and combination particularly pointed out in the appended claims.

To achieve the objects and in accordance with the purposes of the present invention, oxygen-binding proteins are set forth which are capable of stoichiometric reaction with oxygen. To further achieve the objects and in accordance with the purposes of the present invention, as embodied and broadly described herein, portable DNA sequences coding for hemoglobin proteins are provided.

Particularly preferred for expression in bacteria, portable sequences are provided which code for the hemoglobin of the filamentous bacterium Vitreoscilla. These sequences comprise nucleotide sequences capable of directing intracellular production of oxygen-binding proteins. The portable sequences may be either synthetic sequences or restriction fragments ("natural" DNA sequences).

To facilitate identification and isolation of natural DNA sequences for use in the present invention, the inventors have developed a Vitreoscilla genomic library. This library contains the genetic information capable of directing a cell to synthesize the hemoglobin of the present invention. Other natural DNA sequences which may be used in the recombinant DNA methods set forth herein may be isolated from other genomic libraries.

Additionally, portable DNA sequences useful in the processes of the present invention may be synthetically created. These synthetic DNA sequences may be prepared by polynucleotide synthesis and sequencing techniques known to those of ordinary skill in the art.

Additionally, to achieve the objects and in accordance with the purposes of the present invention, a recombinant-DNA method is disclosed which results in manufacture by a host cell or microorganism of the instant oxygen-binding proteins using the portable DNA sequences referred to above. This recombinant-DNA method comprises:

(a) preparing a portable DNA sequence capable of directing a host cell to produce a protein having oxygen-binding activity, including hemoglobin activity;

(b) cloning the portable DNA sequence directly into a host cell, or into a vector capable of being transferred into and replicating in a host cell, such vector containing operational elements for the portable DNA sequence;

(c) transferring the vector, if one is used, containing the portable DNA sequence and operational elements into a host cell capable of expressing the oxygen-binding protein; and

(d) culturing the host cell under conditions appropriate for replication and propagation of the vector and expression of the protein; and

(e) in either order:

(i) harvesting the protein; and

(ii) permitting the protein to assume an active structure whereby it possesses oxygen-binding activity.

Additionally, to achieve the objects and in accordance with the purposes of the present invention, recombinant-DNA methods are disclosed which subject to external control the translation and transcription of gene products by a host cell or microorganism using the portable DNA sequences referred to above.

Processes of the invention include methods for subjecting the expression of a selected DNA sequence in a living cell or virus to regulation by oxygen level through the site-specific insertion of promoter/regulator DNA sequences responsive thereto. Also disclosed are improvements in prior methods for securing expression of a selected "foreign" or exogenous sequence in a host microorganism wherein the DNA sequence is stably incorporated as chromosomal or extrachromosomal constituent of the host. Such improvements comprise fusing to the selected DNA sequence a promoter/regulator DNA sequence capable of selectively promoting or inhibiting expression of the selected DNA in response to variations in environmental concentration of oxygen.

To further accomplish the objects and in further accord with the purposes of the present invention, cloning vectors are provided comprising at least one portable DNA sequence. In particular, plasmid pUC19/pRED2 is disclosed.

It is understood that both the foregoing general description and the following detailed description

are exemplary and explanatory only and are not restrictive of the inventi n, as claimed.

The accompanying drawing, which is incorporated in and constitutes a part of this specification, illustrates one embodiment of the invention and, together with the description, serves to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a partial restriction map of the plasmid pUC19/pRED2.

Figs. 2a and 2b are partial restriction maps of plasmids pWLD 5 and pWLD 10, respectively.

Fig. 3 is a partial restriction map of plasmid pBHb3.

Figs. 4A, B, and C and 5A, B, and C show the total cell number, total t-PA and t-PA produced/10^6 cells according to Example 18.

Fig. 6 is a partial restriction map of plasmid pMSG.

Fig. 7 is a graph of results described in Example 37.

BEST MODES FOR CARRYING OUT THE INVENTION

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the drawings and the following examples, serve to explain the principles of the invention.

It must be understood that the present inventors have prepared oxygen-binding proteins in culture by recombinant DNA methods. While some methods for the production and use of these recombinant products are

describ d below, the end use of these products alone is within the scope of the present invention.

As n ted above, the present invention relates in part to portable DNA sequences capable of directing intracellular production of oxygen-binding proteins in a variety of host cells and host microorganisms. "Portable DNA sequence" in this context is intended to refer either to a synthetically produced nucleotide sequence or to a restriction fragment of a naturally occurring DNA sequence. For purposes of this specification, "oxygen-binding protein" is intended to mean a protein with a primary structure as defined by the codons present in the deoxyribonucleic acid sequence which directs intracellular production of the amino acid sequence, and which may or may not include post-translational modifications. It is contemplated that such post-translational modifications include, for example, association with a heme prosthetic group. It is further intended that the term "oxygen-binding protein" refers to either the form of the protein as would be excreted from a cell or as it may be present in the cell from which it was not excreted.

In a preferred embodiment, the portable DNA sequences are capable of directing intracellular production of hemoglobin. In a particularly preferred embodiment, the portable DNA sequences are capable of directing intracellular production of a hemoglobin biologically equivalent to that previously isolated from the filamentous bacterium, Vitreoscilla. By "biologically equivalent", as used herein, it is meant that a protein, produced using a portable DNA sequence of the present invention, is capable of binding oxygen in the same fashion, but not necessarily to the same degree, as the homodimeric

soluble heme protein (subunit MW 15,775) isolable from Vitreoscilla.

As noted above, the present invention also relates in part to portable DNA sequences which contain promoter/regulators which are capable of directing intracellular expression of endogenous or exogenous gene products, in a variety of host cells and host microorganisms. "Portable DNA sequence" and "promoter/regulator" in this context are intended to refer either to a synthetically produced nucleotide sequence or to a restriction fragment of a naturally occurring DNA sequence.

The portable DNA sequences of the present invention may also include DNA sequences downstream from a promoter/regulator which code for at least one foreign protein. For purposes of this specification, "foreign protein" is intended to mean a protein with a primary structure as defined by the codons present in the deoxyribonucleic acid sequence which directs intracellular production of the corresponding amino acid sequence, and which may or may not include post-translational modifications. It is further intended that the term "foreign protein" refers to either the form of the protein as it would be excreted from a cell or as it may be present in the cell from which it was not excreted. The foreign proteins include oxygen-binding proteins such as hemoglobins, leghemoglobins, myoglobins, hemoproteins, hemocyanias, hemerythrins and the like.

In a particularly preferred embodiment, the promoter/regulator contains transcription and translation initiation and control sequences substantially equivalent to those for directing intracellular production of a hemoglobin protein

biologically equivalent to that previously isolated from the filamentous bacterium, Vitreoscilla. By "substantially equivalent", as used herein, is meant that a promoter/regulator perates to express a downstream gene product upon reduction of the level of oxygen available to the host cell below some critical value.

It is of course intended that the promoter/regulators of the present invention may control and initiate transcription and translation of an unlimited number of endogenous and/or exogenous foreign proteins.

A first preferred portable DNA sequence for the promoter/regulators of the present invention contains at least a portion of SEQUENCE ID No. 1, a nucleotide sequence, which reads 5' to 3' and includes the translation initiation sequence ATG and some of the nucleotide sequence of the Vitreoscilla structural gene

The SEQUENCE ID No. 1 exhibits homology with certain sequences which are highly conserved in a variety of promoter/regulators. Using conventional numbering, with the underlining showing the homology in the SEQUENCE ID No. 1 to the consensus sequence, the -10 consensus sequence or Pribnow box sequence is TATAAT(A/G). The -35 consensus sequence is TTGACA, and the consensus Shine-Dalgarno sequence is AGGAGGTXXX(XX)ATG.

In a preferred embodiment, the SEQUENCE ID No. 1 is operatively fused with at least a portion of a downstream sequence of nucleotides which code for at least a portion of the Vitreoscilla hemoglobin protein which contains at least a portion of the amino acid sequence of SEQUENCE ID No. 2

The SEQUENCE ID No. 2 is disclosed in Wakabayashi et al., supra, Nature 322:483, 1986. It is presently believed that the pr tein purified and prepared through the practice of this invention will exhibit a homology of over 80% with this sequence. The protein of this invention has been observed to enhance functioning of a cell in low oxygen environments.

It must be borne in mind in the practice of the present invention that the alteration of some amino acids in a protein sequence may not affect the fundamental properties of the protein. Therefore, it is also contemplated that other portable DNA sequences, both those capable of directing intracellular production of identical amino acid sequences and those capable of directing intracellular production of analogous amino acid sequences which also possess oxygen-binding activity, are included within the ambit of the present invention.

It must also be borne in mind in the practice of the present invention that the alteration of some nucleotide bases in a DNA sequence may not affect the fundamental properties of the coding sequence. Therefore, it is also contemplated that other analogous portable DNA promoter/regulator sequences which are operable through changes in oxygen level are included within the ambit of the present invention.

It is contemplated that some of these analogous amino acid sequences will be substantially homologous to native Vitreoscilla hemoglobin while other amino acid sequences, capable of functioning as oxygen-binding proteins, will not exhibit substantial homology to native Vitreoscilla hemoglobin. By "substantial

homology" as used herein, is meant a degree of homology to native Vitreoscilla hemoglobin in excess of 50%, preferably in excess of 80%.

Similarly, it is contemplated that some of these analogous DNA sequences will be substantially homologous to the sequence set forth above, while other DNA sequences, capable of functioning as the promoter/regulator described above, will not exhibit substantial homology to the sequence outlined above.

As noted above, the portable DNA sequences of the present invention may be synthetically created, by hand or with automated apparatus. It is believed that the means for synthetic creation of these polynucleotide sequences are generally known to one of ordinary skill in the art, particularly in light of the teachings contained herein. As examples of the current state of the art relating tc polynucleotide synthesis, one is directed to Maniatis et al., Molecular Cloning--A Laboratory Manual, Cold Spring Harbor Laboratory (1984), and Horvath et al. An Automated DNA Synthesizer Employing Deoxynucleoside 3'-Phosohoramidites, Methods in Enzymology 154:313-326, 1987, hereby incorporated by reference.

Additionally, the portable DNA sequence may be a fragment of a natural sequence, i.e., a fragment of a polynucleotide which occurred in nature and which has been cloned and expressed for the first time by the present inventors. In one embodiment, the portable DNA sequence is a restriction fragment isolated from a genomic library. In this preferred embodiment, the genomic library is created from the bacterium Vitreoscilla. In other alternative embodiments, the portable DNA sequence is isolated from other genomic and cDNA libraries.

While it is envisioned that the portable DNA sequences of this invention may desirably be inserted directly into the host chromosome, the present invention also provides a series of vectors, each containing at least one of the portable DNA sequences described herein. It is contemplated that additional copies of the portable DNA sequence may be included in a single vector to increase a host cell's ability to produce large quantities of the desired oxygen-binding protein. It is also envisioned that other desirable DNA sequences may also be included in the vectors of this invention. Further, the invention may be practiced through the use of multiple vectors, with additional copies of at least one of the portable DNA sequences of this invention and perhaps other desirable DNA sequences.

In addition, the cloning vectors within the scope of the present invention may contain supplemental nucleotide sequences preceding or subsequent to the portable promoter/regulator and/or DNA sequence. These supplemental sequences are those that will not adversely interfere with transcription of the portable promoter/regulator and/or any fused DNA sequence and will, in some instances, enhance transcription, translation, posttranslational processing, or the ability of the primary amino acid structure of the resultant gene product to assume an active form.

A preferred vector of the present invention is set forth in Figure 1. This vector, pUC19/pRED2, contains the preferred nucleotide sequence which codes for the amino acids set forth above. Vector pUC19/pRED2 cells are on deposit in the American Type Culture Collection ("ATCC") in Rockville, Maryland under Accession No. 67536.

A preferred nucleotide sequence encoding the Vitreoscilla hemoglobin protein and adjacent Vitreoscilla sequences described above is identified in Figure 1 as region A. The above nucleotide sequence reads counter-clockwise through region A of Figure 1. Plasmid pUC19/pRED2 may also contain supplemental nucleotide sequences preceding and subsequent to the preferred DNA sequence in region A, such as terminators, enhancers, attenuators and the like. For proteins to be exported from the intracellular space, at least one leader sequence and any other DNA sequences necessary or preferred for appropriate transcription and subsequent translation of the vector DNA may be included within the scope of this invention.

In a preferred embodiment, cloning vectors containing and capable of expressing the portable DNA sequence of the present invention contain various operational elements in addition to or instead of the promoter/regulator disclosed and claimed herein. These "operational elements" may include at least one promoter, at least one sequence that acts as expression regulator, and at least one terminator codon, at least one leader sequence, and any other DNA sequences necessary or preferred for appropriate transcription and subsequent translation of the vector DNA.

Additional embodiments of the present invention are envisioned as employing other known or currently undiscovered vectors which would contain one or more of the portable DNA sequences described herein. In particular, it is preferred that these vectors have some or all of the following characteristics: (1) possess a minimal number of host-organism sequences; (2) be stable in the desired host; (3) be capable of

being present in a high copy number in the desired host; (4) possess a regulatable prom ter; and (5) have at least one DNA sequence coding f r a selectable trait present on a portion of the plasmid separate from that where the portable DNA sequence will be inserted. Alteration of vectors to meet the above criteria are easily performed by those of ordinary skill in the art in light of the available literature and the teachings herein. It is to be understood that additional cloning vectors may now exist or will be discovered which have the above-identified properties and are therefore suitable for use in the present invention and these vectors are also contemplated as being within the scope of this invention.

As set forth in Example 1, an E. coli vector system is a preferred embodiment. Various cloning vehicles are required for the range of host cells and organisms suitable for insertion of the portable DNA sequences of the present invention, as set forth below. In light of the available literature, choice of such a cloning vehicle, if necessary, is within the ordinary skill in the art.

Additional bacterial hosts are suitable, including, without limitation: bacteria such as members of the genera Bacillus, Pseudomonas, Alcaligenes, Streptococcus, Lactobacillus, Methylophilus, Xanthomonas, Corynebacterium, Brevibacterium, Acetobacter, and Strepotomyces.

Examples of suitable eucaryotic host microorganisms would include fungi, yeasts such as Saccharomyces and Candida, and molds such as Aspergillus, Pennicillium, Trichederma and Cephalosporium (Acremonium).

It is envisioned that the scope of this invention would cover expression systems in eucaryotic microorganisms and host cultured cells derived from multicellular organisms, including animals, insects and plants, which are grown in the presence of oxygen. The promoter/regulator of the present invention is especially useful in a host which switches from low to very high expression activity upon reduction of dissolved oxygen concentration in the medium. Such expression systems need not be derived from Vitreoscilla.

Various vector systems will be suitable for these and other desirable hosts, including plasmids, viruses and bacteriophages. The following, noninclusive list of cloning vectors is believed to set forth vectors which can easily be altered to meet the above criteria and are therefore preferred for use in the present invention. Such alterations are easily performed by those of ordinary skill in the art in light of the available literature and the teaching herein.

For example, many selectable cloning vectors have been characterized for use in E. coli, including pUC8, pUC9, pBR322, pGW7, placI^q, and pDP8, Maniatis et al., supra. A bifunctional vector that replicates in E. coli and can also be used in Streptomyces is pKC462a. Suitable vectors for use in Bacillus include: pUB110, pSA0501, pSA2100, pBD6, pBD8, and pT127, Ganesan and Hock, eds., Genetics and Biotechnology of Bacilli, Academic Press 1984. In Pseudomonas, RSF1010, Pms149, pXT209, and RK2 are suitable; some of these vectors are useful in a wide range of gram-negative bacteria including Agrobacterium and Xanthomonas. For Saccharomyces, it is possible to use YEp24, YIp5, and YRp17, Botstein

and Davis, Molecular Biology of the Yeast Saccharomyces (Strathern et al., eds), Cold Spring Harbor Laboratory, 1982. In mammalian systems retrovirus vectors such as those derived from SV40 are typically used.

Synthesis and/or isolation of necessary and desired component parts of cloning vectors, and their assembly is believed to be within the duties and tasks performed by those with ordinary skill in the art and, as such, are capable of being performed without undue experimentation.

In construction of the cloning vectors of the present invention, it should additionally be noted that multiple copies of the promoter/regulator with any fused gene sequences and/or of the portable DNA sequence coding for the oxygen-binding protein and its attendant operational elements as necessary may be inserted into each vector. In such an embodiment, the host organism would produce greater amounts per vector of the desired oxygen-binding protein. The number of multiple copies of the DNA sequence which may be inserted into the vector is limited only by the ability of the resultant vector, due to its size, to be transferred into and replicated and expressed in an appropriate host.

Additionally, it is preferred that the cloning vector contain a selectable marker, such as a drug resistance marker or other marker which causes expression of a selectable trait by the host. In a particularly preferred embodiment of the present invention, the gene for ampicillin resistance is included in vector pUC19/pRED2. Such a drug resistance or other selectable marker is intended in part to facilitate in the selection of transformants.

Additionally, the presence of such a selectable marker on the cloning vector may be of use in keeping contaminating microorganisms from multiplying in the culture medium. In this embodiment, such a pure culture of the transformed host organisms would be obtained by culturing the organisms under conditions which require the induced phenotype for survival.

It is noted that the portable DNA sequence of the present invention may themselves be used as a selectable marker, in that they provide enhanced growth characteristics in low oxygen circumstances, and also engender an easily visible reddish tint in the host cells.

The promoter/regulators of this invention are capable of controlling expression of proteins or, thereby, of controlling synthesis of metabolites normally made by a cell, or of natural or unnatural metabolites and proteins expressed in a cell via genetic manipulation. This would include heterologous proteins--either intracellular or extracellular--as well as biopolymers such as polysaccharide materials, simpler metabolites such as amino acids and nucleotides, antibiotics and other chemicals produced by living cells or cellular biocatalysts.

The oxygen-binding proteins of the present invention, prepared by the recombinant-DNA methods set forth herein, will enable increased research into the growth of organisms in oxygen-poor environments. In addition, the oxygen-binding proteins of the present invention are useful in enhancing oxygen supply to cells or in other oxygen-utilizing processes (Adlercreutz et al., Biocatalyst in Organic Synthesis, Symposium of the Working Party on Immobilized Biocatalysts of the European Federation

of Biotechnology, Abstracts, p.18, 1985), and for binding and separating oxygen from other fluids or gases (Bonaventura et al., Underwater Life Support Based on Immobilized Oxygen Carriers, Applied Biochemistry and Biotechnology 9:65-80, 1984). Furthermore, the oxygen-binding proteins of this invention are capable of increasing production of cells, or of proteins or metabolites normally made by a cell, or of natural or unnatural metabolites and proteins expressed in a cell via genetic manipulation. This would, as described above, include heterologous proteins, biopolymers, simpler metabolites, antibiotics, and other chemicals produced by living cells or cellular biocatalysts.

The protein products of this invention also have applications as diverse as enhancing certain oxygen-requiring steps in fermentation, enzymatic degradation, toxic chemical waste treatment, brewing and particular oxidative reactions and transformations such as steroid conversions.

This invention also relates to a recombinant-DNA method for the production of oxygen-binding proteins. Generally, this method includes:

(a) preparing a portable DNA sequence capable of directing a host cell or microorganism to produce a protein having oxygen-binding activity;

(b) transferring the portable DNA sequence directly into the host, or cloning the portable DNA sequence into a vector capable of being transferred into and replicating in a host cell or microorganism, such vector containing

operational elements for the portable DNA sequence;

(c) transferring the vector containing the portable DNA sequence and operational elements into a host cell or microorganism capable of expressing the oxygen-binding protein;

(d) culturing the host microorganism under conditions appropriate for replication and propagation of the vector and/or expression of the protein.

In this method, the portable DNA sequences are those synthetic or naturally-occurring polynucleotides described above. In a preferred embodiment, the portable DNA sequence codes for at least a portion of the Vitreoscilla hemoglobin protein are described above.

This invention also relates to a recombinant-DNA method for the use of these promoter/regulators. Generally, this method provides a process for subjecting the expression of a selected DNA sequence to external control under given environmental conditions which comprises the steps of:

(a) providing at least one selected isolated structural gene that is transcriptionally and/or translationally responsive to a Vitreoscilla hemoglobin promoter/regulator DNA sequence under the given environmental conditions; and

(b) operatively fusing the selected structural gene with said promoter/regulator DNA sequence.

It is envisioned that the portable DNA sequences may be inserted directly into the host chromosome, or alternatively may utilize a vector cloning system. The vectors contemplated as being useful in the present method are those described above. In a preferred embodiment, the cloning vector pUC19/pRED2 is used in the disclosed method.

A vector thus obtained may then be transferred into the appropriate host cell or organism. It is believed that any microorganism having the ability to take up exogenous DNA and express those genes and attendant operational elements may be chosen. Particular hosts which may be preferable for use in this invention include those described above. Methods for transfer of vectors into hosts are within the ordinary skill in the art. For ultimate expression in certain microorganisms such as yeast, it may be desirable that the cloning vector be first transferred into another microorganism such as Escherichia coli, where the vector would be allowed to replicate and from which the vector would be obtained and purified after amplification, and then transferred into the yeast for ultimate expression of the oxygen-binding protein.

The host cells or microorganisms are cultured under conditions appropriate for the expression of the oxygen-binding protein. These conditions are generally specific for the host organism, and are readily determined by one of ordinary skill in the art, in light of the published literature regarding the growth conditions for such organisms.

In one embodiment for expressing globins, conditions necessary for the regulation of the expression of the DNA sequence, dependent upon any operational elements inserted into or present in the vector, would be in effect at the transformation and culturing stages. The cells are grown to a high density in the presence of appropriate regulatory conditions which inhibit the expression of the DNA sequence. When optimal cell density is approached, the environmental conditions are altered to those appropriate for the expression of the portable DNA sequence. It is thus contemplated that the production of a cloned protein will occur in a time span subsequent to the growth of the host cells to near optimal density, and that the resultant cloned protein product would be harvested, if desired, at some time after the regulatory conditions necessary for its expression were induced.

Where the operational elements used are in the promoter/regulator sequence of this invention, these conditions are as follows. The cells are grown to a high density in the presence of appropriate levels of oxygen which inhibit the expression of the DNA sequence. When optimal cell density is approached, the environmental oxygen level is altered to a lower value appropriate for the expression of the portable DNA sequence. Levels from less than about 1% oxygen-saturation to an oxygen saturated solution are within the scope of this invention. It is thus contemplated that the production of any desired fused product will occur in a time span subsequent to the growth of the host cells to near optimal density, and that the resultant product would be harvested, if desired, at some time after the oxygen level necessary for its expression were reached.

If harvesting of the oxygen-binding protein products of the present invention is desired, it may be done prior or subsequent to purification and prior or subsequent to assumption of an active structure.

It is currently believed that some percentage of the oxygen-binding proteins of the present invention will assume their proper, active structure upon expression in the host cell or organism. If desired, the oxygen-binding protein may be transported across a cell membrane. This will generally occur if DNA coding for an appropriate leader sequence has been linked to the DNA coding for the recombinant protein. The structures of numerous signal peptides have been published. It is envisioned that these leader sequences, included in or added to at least some portion of the portable DNA as necessary, will direct intracellular production of a fusion protein which will be transported through the cell membrane and will have the leader sequence cleaved upon release from the cell.

Additional uses of the oxygen-binding proteins of the present invention are envisioned. The purified proteins and/or the whole cells and/or extracts of the cells of the present invention themselves may be used to bind to oxygen or proteins and thus could function somewhat as erythrocytes.

The present invention may also be used as a method for transporting and enhancing oxygen supply to cells or in other oxygen-utilizing processes by delivering the oxygen-binding proteins--isolated in lysates and crude cell preparations, purified from extracts, in synthetic sequences, or in whole cells containing the proteins--where desired. It is envisioned that the protein products of the present invention could

valuably be added to media for culturing cells and thereby enhance the transport of oxygen.

It is also envisioned that the proteins of the present invention may be used for binding and separating of oxygen from fluids such as seawater and from other gases.

It is understood that application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use and manufacture appear below.

INDUSTRIAL APPLICABILITY

The products and processes of the present invention find usefulness in a range of medical, laboratory and industrial applications. The invention provides metabolically engineered cells with enhanced growth characteristics for increasing production of various proteins or metabolites by those cells. The invention further provides a method for subjecting expression of a certain DNA sequence to external control under given environmental conditions. Also provided are recombinant-DNA fusion gene products, expression vectors, and nucleotide base sequences for the practice of the invention. The products and processes of the present invention find applications in a range of aerobic processes, such as manufacture of cloned proteins and synthesis of metabolites, chemical production by fermentation, enzymatic degradation, waste treatment, brewing and a range of oxidative reactions.

EXAMPLES

EXAMPLE 1 - CLONING AND EXPRESSION OF HEMOGLOBIN FROM VITREOSCILLA IN ESCHERICHIA COLI.

Materials and Methods. Vitreoscilla sp. (Murray strain no. 389) was obtained from Dr. Webster (Department of Biology, Illinois institute of Technology, Chicago, Illinois 60616, USA), and grown in a medium containing 1.5% yeast extract, 1.5% peptone, and 0.02% sodium acetate (pH 8.0 with NaOH). This strain is also available from ATCC, accession number 13981.

E. coli JM101 were obtained from the laboratory of Dr. Simon (Division of Biology, California Institute of Technology, Pasadena, California 91125, USA), and grown in L broth containing 1% Bactotryptone, 0.5% yeast extract and 1% sodium chloride. This strain is also available from ATCC, accession number 33876.

Plasmid pUC19 (Yanisch-Perron et al., Improved M13 phage cloning vectors and host strains: nucleotide sequences of m13mp18 and pUC19 vectors, Gene 33:103-109, 1985) packaging kits were purchased from Pharmacia. All restriction enzymes, T4 polynucleotide kinase and T4 ligase were from New England Biolabs or Bethesda Research Laboratories. Calf intestine alkaline phosphatase was from Pharmacia. Mixed oligonucleotide probes were synthesized with an Applied Biosystems synthesizer. Kodak XAR5 x-ray film was used for autoradiography. Geneclean kits were purchased from Bio101. All other chemicals were of analytical grade.

Vitreoscilla genomic DNA was isolated according to the protocol of Silhavy et al., Experiments with gene fusions, Cold Spring Harbor Laboratory (1984),

specifically incorporated herein. HindIII-digested Vitreoscilla DNA was ligated into the phosphatased HindIII site of pUC19 and transf rm d into JM101. Recombinant colonies and plaques were transferred on nitrocellulose filters as described in Maniatis, et al., Molecular cloning--a laboratory manual, Cold Spring Harbor Laboratory (1982) and specifically incorporated herein. Rapid plasmid isolation from recombinant colonies were done according to Silhavy et al., supra. Digested fragments of plasmid DNA or fractions of genomic DNA were isolated from agarose gels using Geneclean kits. E. coli cells were transformed by the $CaCl_2$ method of Silhavy et al., supra. Plasmid uptake was induced by heat-shocking chilled competent cells at 37°C for 5 minutes.

For Southern hybridizations the reagents suggested in Dupont catalog No. NEF-976, Protocols for electrophoretic and capillary transfer of DNA and RNA, DNA and RNA hybridization, and DNA and RNA rehybridization (1985), specifically incorporated herein, were used, whereas for colony and plaque hybridizations those described in Maniatis et al., supra, were used. Filters were prehybridized at 45-50°C for 2-4 hours and hybridized at 30°C for 20-24 hours. 200 picomoles oligonucleotide kinased with 200 microCi (^{32}P)ATP (sp. act. 7000 Ci/mmol) were used as probe. Filters were washed in 2 X SSC, 0.1% SDS at room temperature (3 X 5 minutes) and at 46°C (for the C-terminal probe) and 50°C (for the N-terminal probe) prior to autoradiography.

SDS-polyacrylamide gel electrophoresis was done according to standard protocols, Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227:580-685, 1970, specifically incorporated herein, with a 12.5%

resolving gel. Protein in the gel was visualized by the silver staining method or Merril et al., Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins, Science 211:1437-1438, 1983.

Results. Three sets of mixed oligonucleotide probes were synthesized which had a predicted homology to two domains in the hemoglobin gene, one N-terminal and one C-terminal. A pUC19-HindIII library of Vitreoscilla DNA was test-plated on rich plates with ampicillin, X-gal and IPTG. More than 70% of the colonies were probable recombinants, as estimated by visual inspection. About 10,000 colonies were then screened. Three positives were identified. Because of the high density of colonies on the plate, these, along with their immediate clones from each group, were assayed by rapid isolation and HindIII digestion. One of these, pRED1, had three inserted fragments including a 2.2kb one. Subsequent digestion of this plasmid with various endonucleases and Southern hybridization of the resulting DNA bands did confirm that the 2.2kb band did indeed contain the entire hemoglobin gene, since no HindIII sites are expected to exist upstream or downstream of the regions spanned by the oligomeric probes.

The HindIII fragment from pRED1 that contained the hemoglobin structural gene was purified and reinserted by standard protocols into pUC19 in both orientations (pRED2 and pRED3). E. coli cells containing plasmids pRED1, pRED2, pRED3 and pUC19 as well as Vitreoscilla cells were grown to stationary phase and cell extracts were assayed on an SDS-polyacrylamide gel for the existence of the hemoglobin polypeptide. The hemoglobin was expressed as a major cellular protein in all recombinant cells.

Since both plasmids PRED2 and PRED3 express ab ut equal amounts of this polypeptide, it is presently believed that the gene is probably expressed from its natural promoter in E. coli.

The restriction map of plasmid pRED2 is shown in Figure 1.

Determination of the sequence of the relevant region of the fragment isolated from the Vitreoscilla Ugenomic library was accomplished as follows:

The HindIII-SphI fragment from plasmid pRED2 which contains the structural gene and adjacent sequences was subcloned into pUC19 (purchased from Bethesda Research Labs) to obtain plasmid pRED4. An MluI site was identified, by restriction mapping the resulting plasmid, which breaks up the HindIII-SphI insert into two fragments which were individually sequenced using conventional protocols (Maxam and Gilbert, Sequencing end-labeled DNA with base-specific chemical cleavages, Methods in Enzymology 65:499-560, 1980; Iverson and Dervan, Adenine specific DNA chemical sequencing reaction, Nuclear Acids Research 15:7823-7830, 1987).

The nucleotide sequence of the important portion of the HindIII-SphI fragment includes a putative E. coli promoter, ribosome binding site, the complete VHb structural gene (start and stop codons are underlined) and a putative E.- coli transcription terminator (Khosla and Bailey, The Vitreoscilla hemoglobin gene: molecular cloning nucleotide sequence and genetic expression in Escherichia coli, Mol. & Gen. Genet., 214:158-161 (1988).

EXAMPLE 2 - GROWTH ENHANCEMENT IN E. COLI WITH pPRED2: SHAKE FLASK CULTURES.

In this Example, the growth behavior of E. coli cells producing active Vitreoscilla hemogl bin was compared to that of control strains grown under identical conditions. The following strains were studied: (1) JM101:pRED2; (2) JM101:pUC9; and (3) JM101. Plasmids pUC9 and pUC19 are essentially identical except for a difference in one restriction site unrelated to the insert or to any of the functional properties of the plasmid.

Experimental protocol: Cells were grown at 37°C in a complex medium containing 1% (W/V) bactotryptone, 0.5% (W/V) yeast extract, 0.5% (W/V) NaCl, 0.3% (W/V) K_2HPO_4 and 0.1% (W/V) KH_2PO_4 (pH 7.0). Plasmid-containing cells were grown in the presence of 100 mg/L ampicillin. In each case the shake-flask was inoculated with a 1% (V/V) dose of concentrated nutrient broth containing 430 g/L glucose, 5 g/L yeast extract, 110 g/L $(NH_4)_2SO_4$, 8 g/L $MgSO_4 \cdot 7H_2O$, 0.27 g/L $FeCl_3 \cdot 6H_2O$, 0.02 g/L $ZnCl_2 \cdot 4H_2O$, 0.02 g/L $CaCl_2 \cdot 2H_2O$, 0.02 g/L $NaMoO_4 \cdot 2H_2O$, 0.01 g/L $CuSO_4 \cdot 5H_2O$, 0.005 g/L H_3BO_{31} 0.1% (V/V) conc. HCl, 4.2 mg/L riboflavin, 54 mg/L pantothenic acid, 60 mg/L folic acid. This formulation has been successfully used on a previous occasion to grow stationary cells to a high density in a fedbatch mode. The cells were then allowed to grow further until stationary phase was reached again. Optical density was measured at 600 nm on a Bausch & Lomb Spectronic 21 spectrophotometer. Dry weights were measured by spinning 10 mL samples at 4°C, washing once with distilled water and subsequently drying the resuspended sample at 100°C overnight. The heme content of the cells was assayed according to the method of Lamba & Webster (Lamba & Webster, Effect of growth conditions on yield and heme content of Vitreoscilla, Journal of Bacteriology

142:169-173, 1980), and the hem globin activity was measured by the method of Webster & Liu (Webster and Liu, Reduced nicotinamide adenine dinucleotide cytochrome o reductase associated with cytochromic o purified from Vitreoscilla, Journal of Biological Chemistry, 249:4257-4260, 1974.).

Results. The growth properties, heme content and hemoglobin activity of the three strains are documented in the Table below.

		JM101:pRED2	JM101:pUC9	Jm101
1.	OD_{600} before nutrient replenishment	0.937	0.737	0.945
2.	OD_{600}	1.230	0.880	0.985
3.	max. attained dry wt.	1.5g/L	0.85g/L	1g/L
4.	relative heme content	5.5	1	**
5.	relative hemoglobin activity	5	I	**
6.	specific growth rate*	0.04/h	0.01/h	0.009/h

*mean value following additional feeding of shake-flasks as described above

**not assayed

EXAMPLE 3 - GROWTH ENHANCEMENT OF E. COLI WITH pRED2.

A typical high-cell density fermentation is of a fedbatch type. The optimal rate of nutrient addition, and consequently the productivity, is ultimately limited by the rate at which cells can aerobically catabolize the carbon source without generating growth-inhibitory metabolites such as acetate and lactate (Zabriskie and Arcuri, Factors influencing productivity of fermentations employing recombinant microorganisms, Enzyme and Microbial Technology 8:706-717, 1986; Tsai et al, The effect of organic nitrogen and glucose on the productivity of recombinant insulin-like growth factor in high cell density Escherichia coli fermentations, Journal of Industrial Microbiology 2:181-187, 1987). In this experiment, we compare the growth properties of the

recombinant strain (JM101:pRED2) expressing Vitreoscilla hemoglobin with similar plasmid-containing (JM101:pUC9) and plasmid-free (JM101) strains under typical fed-batch fermentation conditions.

Materials and Methods:

Cells were grown in a New Brunswick Microferm fermentor at 37±0.5°C and a pH of 7±0.05 with an initial working volume of 2.5 L. A constant air-flow rate of 4.5 L/min and agitator speed of 300 rpm were maintained throughout each run. Silicone antifoam AF60 was used to control foaming. The batch medium and feed medium 1 listed in Table 2 in Tsai et al, supra were used. Growth following inoculation was in batch mode. After batch stationary phase was reached, continuous feeding was initiated using feed medium 1 at a flow rate of 10 mL/hr. For plasmid-containing cells, 100 mg/L ampicillin was used. In all cases, the dissolved oxygen (DO) levels remained fairly constant around 5% of air saturation for most of the run except during the early log phase and towards batch stationary phase.

Results:

The growth parameters measured for the three strains are listed below. Batch stationary phase refers to conditions before continuous feeding was started.

	JM101:pRED2	JM101:pUC9	JM101:pRED2
Batch log-phase growth rate (h^{-1})	0.95	0.73	0.95
Batch stationary-phase dry cell mass (g/L)	2.6	1.6	2.6
Fed-batch log-phase growth rate (h^{-1})	0.056	0.033	0.066
Final dry cell mass (g/L)	5.8	2.8	5.9

Further, the respiratory behavior of JM101:pRED2 was improved compared to the control strains at low DO levels, as observed in a Gilson respirometer.

Conclusion:

Cells containing Vitreoscilla hemoglobin grow faster and to higher densities than comparable plasmid-containing controls.

EXAMPLE 4 - EXPRESSION OF VITREOSCILLA HEMOGLOBIN (VHb) IN E. COLI UNDER THE REGULATION OF OTHER PROMOTERS.

In Examples 1, 2, and 3 above, the expression of hemoglobin is under the regulation of its native oxygen-regulated promoter. Hence, it is not possible to modulate independently the dissolved oxygen concentration (DO) and the intracellular VHb level. In order to overcome this, the inventors attempted to express this protein under the control of other regulatable promoters which are functional in E. coli, such as trp (Russell and Bennett, Construction and analysis of in vitro activity of E. coli promoter hybrids and promoter mutants that alter

the -35 to -10 spacing Gene 20:231-243, 1982) and tac (deBoer et al, The tac promoter: a functional hybrid derived from the trp and lac promoters Proc. Natl. Acad. Sci. USA 80:21-25, 1983). Materials and Methods:

Plasmid PRED4 was linearized with HindIII and treated with exonuclease Ba131 to generate 5' end deletions in the HindIII-SphI VHb fragment (Maniatis et al, supra). After digestion with SphI, the resulting VHb fragments were cloned into HindIII-SphI digested pUC19. The positions of the deleted end-points were identified by sequencing.

trp and tac promoters and the chloramphenicol acetyl transferase gene (CAT) were purchased from Pharmacia, Inc. Oligonucleotides were synthesized at California Institute of Technology using an Applied Biosystems DNA synthesizer.

All DNA enzymes were obtained from vendors.

The functional assay for the VHb gene product is as described in Webster and Liu, supra.

Cells were pelleted at 4°C and resuspended in 100 mM Tris (pH 7.5), 50 mM NaCl. This cell suspension was sonicated at 75 W for 3 min. on ice. After spinning in at 12,000 g for 10 min., the supernatant was collected and assayed for VHb. Total protein content was estimated using the Bradford assay kit from BioRad Inc. Vhb activity is reported as delta-$A_{419-436}$/mg total protein.

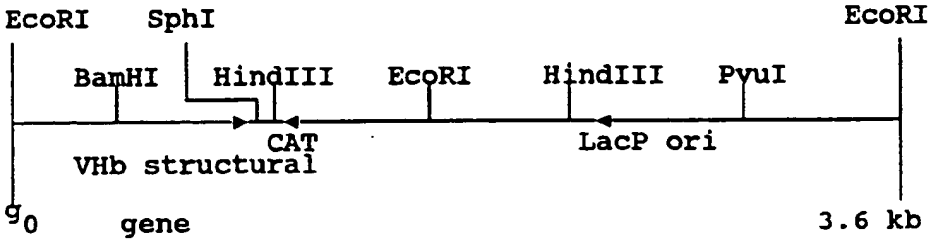
Results:

One of the deletions, pRED302, mapped 2 base-pairs upstream of the ATG start codon for the VHb structural gene. This deletion was used for further work. The EcoRI/BamHI trp promoter cartridge was cloned upstream of the truncated VHb fragment. The following ribosome binding site was synthesized:

5' GATCCCGGGTCTAGAGGA 3'
GGCCCAGATCTCCT

and inserted between the BamHI and nuclease-blunted XbaI sites to give rise to a trp promoter-controlled VHb expression system. The CAT gene (Alton and Vapnek, Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9, Nature 282:864-869, 1979) was inserted downstream and under the control of the lac promoter available on this PUC19-based plasmid. This gene product can be conveniently assayed (Neumann et al, Novel rapid assay for chloramphenicol acetyltransferase gene expression, BioTechniques 5:444-447, 1987) and hence serves as a useful reporter. Finally, the ß-lactamase gene on this PUC19-based plasmid was deleted by digestion and religation with PvuI. The purpose of this step is to eliminate the presence of a plasmid-encoded periplasmic protein. The plasmid thus obtained was called pHbCAT and was transformed into JM101. As a control, the CAT gene was cloned downstream and under the control of the lac promoter in pUC19. The ß-lactamase gene was identically deleted. This plasmid was called pCAT. The restriction maps and the anticipated sequence of relevant regions of these two plasmids are shown below.

pHbCAT (3.6 kb)



EcoRI

5' GAATT CCCCT GTTGA CAATT AATCA TCGAA CTAGT TAACT AGTAC

BamHI

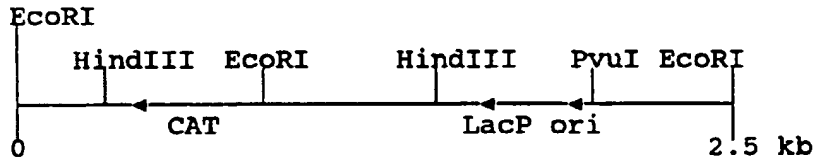
GCAGC TTGGC TGCAG GTCGA CGGAT CCCGG G CTA CAGGA AGTCT

Start codon of VHb

CATGT TAGAC...(same as in Example 2 up to SphI site)

The sequence of the region spanning between EcoRI and the start of the VHb structural gene is shown above. It includes the trp promoter and a synthetic ribosome binding site.

pCAT (2.5 kb)



The effect of tryptophan (repressor) and indole acrylic acid (gratuitous inducer) on VHb levels in JM101/pHbCAT are shown in the Table below. In these experiments, cells were grown to mid-log in minimal

medium containing 3 g/L glycerol, 3 g/L Casamino acids, and the appropriate amount of indole-acrylic aced or tryptophan.

Host: Plasmid:	Tryptophan (mg/L)	Indole-acrylic acid (mg/L)	Specific Hb Activity*
JM101:pCAT	--	--	3.4×10^{-3}
JM101:phbCAT	20	--	6.2×10^{-3}
"	4	--	18.3×10^{-3}
"	--	--	31.5×10^{-3}
"	--	1	29.8×10^{-3}
"	--	2.5	36.5×10^{-3}
"	--	5	47.0×10^{-3}
"	--	10	36.6×10^{-3}

Note *(delta-$A_{419-436}$/mg total soluble protein)

To express the VHb gene under the control of the tac promoter, an expression plasmid plasmid was made using a HindIII-BamHI tac promoter cartridge, the BamHI/SphI fragment from pHbCAT, and the HindIII-SphI digested fragment of the vector pBR322 (Bolivar et al, Construction and characterization of new cloning vehicles. II. A multipurpose cloning system Gene 2:95-113, 1977).

With this construct (pINT1), the level of redness of cells correlated well with varying amounts of the gratuitous inducer IPTG, indicating that the gene product synthesis was under the control of tac. The advantages of this expression system are:

a. Higher expression of vHb, and

b. Ability to use c mplex medium for growth.

EXAMPLE 5 - GROWTH OF E. COLI - EXPRESSING VHb UNDER THE REGULATION OF OTHER PROMOTERS.

The aim of this experiment was to demonstrate the growth effects of VHb on E. coli. In these cases, VHb is expressed using promoters different from the native VHb oxygen-regulated promoter. The strains:plasmids used are:

1. HB101:pBR322 (pBR322 from BRL)

2. JM101:pINT1 (pINT1 discussed in Example 5).

The two hosts have nearly identical genotypes, the major difference being the presence of an F' factor in JM101 which harbors the $lacI^8$ gene. This gene is necessary to keep a strong promoter like tac under control.

The following media recipes shall be henceforth referred to in the appropriate annotated form:

1X LB: 10 g/L Bactotryptone, 5 g/L Yeast Extract, 5 g/L NaCl, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 100 mg/L Ampicillin

2X LB: 20 g/L Bactotryptone, 10 g/L Yeast Extract, 5 g/L NaCl, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 100 mg/L Ampicillin

5X LB: 50 g/L Bactotryptone, 25 g/L Yeast Extract, 5 g/L NaCl, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 100 mg/L Ampicillin.

The experiment was c nducted as follows. Single colonies of the two strains list d above were inoculated into 5 mL 1X LB in a culture tube and grown overnight at 37°C.

0.5mL of the appropriate inoculum was transferred into 250mL culture flasks containing 50 mL medium as follows:

1) HB101:pBR322 : 2X LB
2) HB101:pBR322 : 5X LB
3) JM101:pINT1 : 2X LB
4) JM101:pINT1 : 2X LB + 0.1 mM IPTG
5) JM101:pINT1 : 2X LB# 0.5 mM IPTG
6) JM101:pINT1 : 5X LB
7) JM101:pINT1 : 5X LB# 0.1 mM IPTG
8) JM101:pINT1 : 5X LB# 0.5 mM IPTG

Cells were then grown for 24 h at 37°C in a New Brunswick G24 Environmental Incubator Shaker with the shaker speed adjusted to medium setting. At the end of the experiment, the OD_{600} was measured in a Spectronics 21 spectrophotometer by diluting the culture 10-fold in 1% NaCl. The data are listed below.

Host/Plasmid	LB conc.	IPTC conc. (mM)	Final OD_{600}
HB101:pBR322	2X	0	3.00
JM101:pINT1	2X	0	3.03
JM101:pINT1	2X	0.1	2.91
JM101:pINT1	2X	0.5	3.00
HB101:pBR322	5X	0	2.73
JM101:pINT1	5X	0	3.26
JM101:pINT1	5X	0.1	3.40
JM101:PINT1	5X	0.5	3.15

From this data, the following conclusions may be drawn:

1. In all cases involving 2X LB, the cells grew to approximately the same density. This density is roughly twice that obtained routinely in IX LB under similar growth conditions and indicates exhaustion of available nutrient. In other words, cells have entered stationary phase of growth due to nutrient limitation.

2. It has been demonstrated (Tsai et al., supra) that cells grown in excess nutrient eventually attain an oxygen-limited growth condition due to which they generate inhibitory metabolics such as acetate. Eventually, this leads to cessation of growth, even if more nutrient is supplied. The results of all 5X experiments are indicative of such an occurrence. In other words, oxygen limitation has arisen eventually, causing the culture to reach stationary phase.

3. Hence, it may be argued that under O_2-limited growth, the pres nce of the hemoglobin gene enhances

the growth characteristics of E. coli. This result is similar to that in Examples 3 and 4, with the difference being that here VHb expression is not regulated by DO levels.

4. It appears that under the given growth conditions, there exists an optimal level of VHb expression that maximizes the growth enhancement effect. Such an optimum may be a function of specific growth properties of each cell line and/or plasmid construct as well as of the environmental conditions of growth. The optimum may thus have to be determined for different applications of this technology on a case-by-case basis; however, such determination does not require undue experimentation.

EXAMPLE 6 - EFFECT OF VHb PRESENCE ON THE SYNTHESIS OF ANOTHER CLONED PROTEIN IN E. COLI.

The aim of this experiment was to demonstrate the effect of the VHb gene on the synthesis of a model cloned gene product. This is an important application of the technology, since a wide variety of gene products are produced commercially via recombinant DNA technology. A typical process of this kind involves a high cell density fed-batch fermentation. The productivity of such processes is ultimately limited by insufficient oxygen availability.

The following hosts/plasmids were used in this example:

1. JM101:PCAT
2. JM101:PHbCAT.

The construction of these plasmids is described in Example 5.

The following media compositions were used:

LB: 10 g/L Bactotryptone, 5 g/L Yeast Extract, 5 g/L NaCl, 3 g/L K_2HPO_{41} 1 g/L KH_2PO_4, 30 mg/L Chloramphenicol

10X feed: 100 g/L Bactotryptone, 100 g/L Yeast Extract, 150 mg/L Chloramphenicol.

The experiment was conducted as folLows:

Single colonies of the two strains were inoculated into 5 ml LB in a culture tube and grown overnight. 1 mL of the inoculum was transferred into 100 mL fresh LB and the growth curve was followed. As cells approached the end of log phase, a 1 mL pulse of 10X feed was added and the growth burst was followed. A second pulse was similarly added. At the end of this growth phase, a pulse of 1 mL 10X feed containing 100 mM IPTG was added to induce the expression of the CAT gene. One hour later, a sample was withdrawn for monitoring CAT activity. The results of the experiment are shown below.

	JM101:pCAT	JM101:pHbCAT
1) Klett before IPTG pulse	670	700
2) Total soluble protein (mg/ml culture broth)	0.31	0.435
3) CAT activity (units/mg soluble protein)	1.39×10^4	2.67×10^4
4) CAT activity (units/ml culture broth)		

From the above data, the following conclusions may be drawn.

1. The presence of VHb enhances the synthesis of a cloned gene product, ev n at low l vels of VHb industrion.

2. Besides increasing the amount of cloned gene product per unit volume of culture, the presence of VHb may also enhance the specific activity (activity per unit amount of totally soluble protein) of the cloned gene product.

EXAMPLE 7 - OXYGEN-DEPENDENT REGULATION OF EXPRESSION OF VHb IN E. COLI BY NATIVE VITREOSCILLA HEMOGLOBIN UPSTREAM SEQUENCES.

The aims of this experiment were as follows:

1. To demonstrate that VHb gene expression in E. coli increases in response to decreasing oxygen levels in the medium.

2. To establish transcriptional-level regulation of gene expression.

3. To determine the sensitivity of this oxygen-dependent genetic switch in response to changes in dissolved oxygen concentrations.

Materials and Methods:

The HindIII-SphI fragment containing the VHb gene and flanking sequences was cloned into the corresponding sites of the vector pBR322, thereby creating the plasmid pOX1. This was then transformed into the E. coli hold, HB101. The fermentation was conducted in a New Brunswick Bioflo II fermentor with a 2.5 L working volume using LB (10 g/L Bactotryptone, 5 g/L yeast extract, 5 g/L NaCl, 3 g/L K_2H-PO_4, 1 g/L KH_2PO_4) plus 8 mg/L silicone antifoam as medium at 37°C, pH 7.0 with a constant agitation speed of 300

rpm. All other methods involve conventional protocols (Maniatis, et al., supra).

Cells were grown to an OD_{600} ≈ 0.25 with DO maintained greater than 50% air saturation at all times. At this point, the air supply was gradually reduced so that the DO fell to about 1% air saturation in an almost linear manner over a period of 45 min. (i.e., a time scale long enough for gene induction, yet within approximately one generation time of E. coli). Samples were intermittently taken and analyzed for VHb mRNA and protein levels. Later, nitrogen was sparged in the vessel to study the induction of the VHb promoter under strictly anaerobic conditions.

Results:

1. The level of VHb mRNA increased about ten-fold as DO dropped from 70% to 1% air saturation.

2. There was a corresponding increase in VHb activity. A lag was noticed between increase in VHb mRNA level and increase in the quantity of active VHb. This may occur because of the requirement of additional heme biosynthesis in the host cell in order to produce active VHb.

3. The VHb promoter was switched on to significant levels only below 40% air saturation and attains maximum induction levels below 5% air saturation.

4. The promoter switches off under strictly anaerobic conditions, indicating the importance of a basal level of aerobicity in the environment for maximal gene expression.

EXAMPLE 8 - HETEROLOGOUS EXPRESSION OF VHb IN A EUCARYOTIC SACCHAROMYCES CEREVISIAE HOST.

In this experiment, we attempted to express the VHb gene in a model eucaryote, Saccharomyces cerevisiae. The vector used was pBM 150 (Johnston and Davis, Sequences that regulate the divergent GAL1-GAL10 promoter in Saccharomyces cerevisiae, Molecular and Cellular Biology 4:1440-1448, 1984) and the host strain was D603 (ade$^-$, ura$^-$, met$^-$, lys$^-$, reg 1), Srienc et al., Effect of ARS1 mutations on chromosome stability in Saccharomyces cerevisiae, Molecular and Cellular Biology 5:1676-1684, 1985. The truncated VHb structural gene referred to in Example 4 was cloned downstream of the GAL10 promoter to create plasmid pYRED1.

Results:

The recombinant strain D603:pYRED1 was significantly redder than the control D603:pBM150 when grown in the presence of galactose (2% peptone, 1% yeast extract, 2% galactose). Inocula were grown in minimal galactose medium. Significant hemoglobin activity was determined in sonicates from D603:pYRED1 compared to D603:pBM150 controls based on the difference spectrum hemoglobin analysis referenced in Example 2.

EXAMPLE 9 - GROWTH ENHANCEMENT OF SACCHAROMYCES CEREVISIAE CELLS EXPRESSING VHb.

In this example, the effect of VHb expression on the growth of the yeast Saccharomyces cerevisiae was studied. The VHb gene was cloned into a yeast expression plasmid, AAH5, that is stably maintained as an extrachromosomal plasmid in yeast cells.

Materials and Methods:

Plasmid pEX-2 was constructed as follows. The BamHI/SphI fragment described in Example 4 was cloned by blunt-end ligation into the HindIII site of the

yeast expression vector AAH5 (Ammerer, Expression of genes in yeast using the ADC1 promoter, Methods in Enzym logy 101:192-201, 1983). AAH5 contains the selectable yeast marker Leu 2, the 2 micron circle origin of replication, and a unique HindIII site flanked by the transcriptional promoter and terminator regions of the yeast alcohol dehydrogenase-1 (ADH-1) gene. The ADH-1 promoter will support high levels of transcription of any sequence cloned into the HindIII site. The ADH-1 gene is constitutively expressed in yeast.

S. cerevisiae strain 488-0 (leu2, ura3, his 1-7) was transformed with plasmids AAH5 and PEX-2 by the rapid colony transformation procedure (Keszenman-Pereyra and Heida, A colony procedure for transformation of Saccharomyces cerevisiae, Curr. Genet. 13:21-23, 1988), and plated on synthetic dextrose (SD) medium (Rose, Isolation of genes by complementation in yeast, Methods in Enzymology, 152: 481-504, 1987) without leucine. A representative clonal cell line from each transformation was established after colony purification of a primary transformant.

For the growth studies, single yeast colonies were inoculated into 2 mL of SD -leu (+leu for 488-0) and cultured for 24 hr at 260 rpm at 30°C in a Labline Model 3258 Orbit Enviro-shaker. 0.5 mL of this inoculum was added to 50 mL of the same medium in a 250 mL flask and cultured at 260 rpm at 30°C. Cell growth was measured by turbidity (A600nm) using a Perkin-Elmer Lambda 4A Spectrophotometer. When the glucose level of the culture medium dropped below 2.0 mM, the cultures were pulsed with 1/40 volume of a concentrated medium containing 20 x SD (40% glucose, 13.3% Difco yeast nitrogen base without amino acids, and 1.6 mg/mL of all the amino acids except leucine.

For strain 488-0, 1.6 mg/mL leucine was included in the pulse medium). Gluc s concentration was estimated using Ames Glucostix test strips.

Results:

A comparison of the growth curves Of strains 488-0, 488-0:AAH5, and 488-0:pEX-2 grown under the above conditions revealed the following:

1. All three strains grew at an equivalent rate during the logarithmic stage of growth.

2. The VHb-containing strain 488-0:pEX-2 grew to a final optical density of 13.0, while strains 488-0:AAH5 and 488-0 grew to optical densities of only 10.0 and 9.5, respectively. This represents a 26.0% increase in final cell density between a strain carrying the VHb gene on a plasmid (488-0:pEX-2) compared to a strain containing the identical plasmid without the VHb gene (488-0:AAH5). In addition, this represents a 32.6% increase in the final cell density of 488-0:pEX-2 over the strain containing no AAH5-derived plasmid (488-0).

EXAMPLE 10 - GROWTH ENHANCEMENT DUE TO EXPRESSION OF VHb IN E. COLI FROM A CHROMOSOMICALLY INTEGRATED GENE.

In this example, the tac-VHb gene fusion, discussed in Example 4, was integrated into the chromosome of E. coli MG1655 (obtained from Cold Spring Harbor Laboratory, NY).

Materials and Methods:

A defective Tn10 transposon (Foster, et al., Three Tn10-associated excision events: Relationship to transposition and role of direct and inverted repeats, Cell, 23:215-227, 1981) was constructed as follows. A kanamycin resistance gene (Pharmacia

Inc.) was cloned into the SalI site of PINT1 (Example 5). The EcoRI/EagI fragment from the resulting plasmid, which contains the entire tac-VHb fusion and Kan^R gene, was cloned between the inverted repeats (bases 1-66 on the right end and bases 9234-9300 on the left end) of a Tn10 derivative which lacks the transposase gene (obtained from Cold Spring Harbor Laboratory, NY). The resulting element, Tn10dKan-tac-VHb, was cloned into a multicopy plasmid containing a tac-Tn10 rightward transposase (obtained from Cold Spring Harbor Laboratory, NY). Transposition was induced with 0.5 mM IPTG for 4 hr, following which cells were plated on lactose-MacConkey-Kan plates. Lac-mutants were selected and the transposon-induced mutation was induced into E. coli MG1655 using Pi phage (Silhavy et al., supra). One of the resulting Lac-colonies, which was further purified and checked for Lac^-, Kan^R, Amp^S, VHb^+ (IPTG inducible, as confirmed by assay described in Example 3), was designated GR013. Comparison of growth properties of strains MG1655 and GR013 in 2X LB (described in Example 6) containing 1 mM IPTG, followed by addition of a concentrated feed (25% Bactotryptone, 12.5% yeast extract), showed an increase in final cell densities (final cell densities: OD_{600} = 16.8 for MG1655, OD_{600} = 18.1 for GR013).

EXAMPLE 11 - CONSTRUCTION OF AN EXPRESSION VECTOR FOR CULTURED MAMMALIAN CELLS

The eukaryotic expression vector pMSG (Pharmacia LKB Biotechnology, Piscataway, NJ) was propagated in *E. coli* HB101. *E. coli* HB101 was transformed with pMSG using a standard $CaCl_2$ protocol (1). After culturing transformants overnight, pMSG plasmid DNA was isolated by standard miniprep technique (1). The authenticity of this plasmid was confirmed by

performing several restriction digestions of the original and the HB101-derived pMSG plasmid DNA. A frozen stock of HB101-pRED302 (2) was plated out on a agar plate. The following day, a 10 ml overnight culture was inoculated from a single colony. Miniprep plasmid isolation was carried out on this overnight culture for pRED302 isolation. Plasmid pRED302 was digested with XbaI and SspI (Boerhringer Mannheim, Indianapolis, IN) and the digested sample was run on a 0.7% agarose gel. This resulted in 3 fragments of approximate sizes of 2.5 kb, 0.56 kb and 0.54 kb. Test digestions were carried out with the two smaller fragments using the restriction enzymes MluI and Bsu361 since these sites are present in the VHb structural gene. Electrophoresis of these digested samples on a 2% agarose gel confirmed that the lower band contains the VHb structural gene. The lower band was eluted and purified from the gel using the GeneClean Kit (Bio 101, La Jolla, CA) resuspended in 30 ml TE (pH 8.0) and stored at -20°C. Part of this purified fragment was used for construction of pMSG-VHb and remaining sample was frozen down at -20°C for use in Southern hybridization experiments. The construction of pMSG-VHb was done by utilizing the multicloning site of pMSG. pMSG was sequentially digested with SmaI and NheI with an intermediate phenol:chloroform extraction and ethanol precipitation step. The digested vector was run on a 0.7% agarose gel and the larger fragment was purified using a GeneClean Kit, resuspended in 20 ml TE (pH 8.0) and stored at -20°C. An overnight blunt-sticky end ligation reaction was carried out at 8-15°C for cloning the XbaI/SspI VHb fragment into the NheI/SmaI digested pMSG. This vector was named pMSG-VHb. The following day, competent HB101 were transformed separately with no plasmid, pMSG and pMSG-VHb respectively and spread on LB-ampicillin plates.

Nine colonies were picked from pMSG-VHb plate as potential transformants for further analysis. Minipreps were carried ut on ov rnight cultures of all these potential transformants for pMSG-VHb plasmid DNA isolation. Vectors pMSG and pMSG-VHb were digested with the differed restriction enzymes BamH1, Hing111, Sal1 and M1u1 for verifying the authenticity of the new construct. When run on a 0.7% agarose gel, those samples confirmed the presence of the VHb insert in the multicloning site of pMSG. A maxiprep was carried out using two 100 ml cultures of one of the pMSG-VHb transformants for large-scale plasmid DNA isolation. The DNA was extracted with phenol:chloroform, ethanol precipitated, the pellet resuspended in 1 ml TE (pH 8.0) and stored at -20°C until further use in CHO cell transfection.

EXAMPLE 12 - TRANSFECTION OF CHO CELL WITH AN EXPRESSION VECTOR

A Chinese hamster ovary (CHO) cell line producing tPA (ATCC 9606) was obtained from ATCC (Bethesda, Maryland). These cells were grown routinely in a non-selection medium containing DMEM (high glucose) (GIBCO, Grand Island, NY) supplemented with 1X penicillin-streptomycin-glutamine (Irvine Scientific, Irvine, CA) solution and 5% dialyzed FBS (GIBCO, Grand Island, NY) in a 5% CO_2 humidified incubator at 37°C. Tissue culture dishes (100 & 20mm) were used in all the experiments. The selection medium for transfected cells contains 25 mg/1 mycophenolic acid (Grand Island, NY), 1 X HAT (Gibco, Grand Island, NY) and 250 mg/1 xanthine (Sigma, St. Louis, MO in addition to the non-selections medium components. During regular cultures, cells were passaged every 2-3 days upon semi-confluency. Total cell counts and cell size distribution were monitored by the Coult r

counter. Cell viability was determined using the trypan blue exclusion method.

The CHO cells producing tPA were transfected with pMSG-VHb using the standard calcium phosphate procedure described in Maniatis (1). Briefly, one 100 x 200 mm tissue culture dish with 10 ml non-selection medium was inoculated with 1 x 10^6 cells 24 hours prior to transfection. Twenty mg pMSG-VHb DNA was digested overnight with EcoR1 at 37°C. This linearized vector DNA was subjected to phenol:chloroform extraction and ethanol precipitation and the resulting pellet was resuspended in an appropriate volume of 0.1 X TE (pH 8.0). This was combined with 20 mg of carrier DNA and the calcium phosphate-DNA precipitate was formed according to the standard protocol. 1 ml of this precipitate was added to the 100 mm dish containing cells. These cells were incubated for 24 hours at 37°C upon which the medium was aspirated and fresh non-selection medium added in order to facilitate the expression of XGPRT. Cells were then diluted 1:8 in selection medium and allowed to grow in this medium. The medium was replaced every two days and single colonies began to appear in about two weeks. About 40 colonies resistant to selection pressure were picked using cotton tips wetted with trypsin and transferred to 24-well tissue culture plates. These colonies were expanded to 60 and 100 mm dishes upon reaching confluency in the wells. All except 3 clones were frozen down at -70°C. The three clones (clones 4, 19 and 30) were gradually expanded to 100 mm dish cultures for further studies.

EXAMPLE 13 - VALIDATION OF CLONING OF THE VHb GENE IN ENGINEERED OR RECOMBINANT CHO CELLS

Genomic DNA was isolated from various clones using standard protocos from Maniatis et al. (1). 5 ng DNA each of th parental CHO-tPA and three VHb-CHO clones were digested overnight with EcoR1 at 37°C. Agarose gel electrophoresis of these digested DNAs was carried out using 0.5 X TBE for six hours at 60 volts. The DNA was then transferred overnight to Immobilon-S membrance (Millipore, Bedford, MA) using a capillary blot technique (1). The VHb gene probe isolated by miniprep was labeled with biotin using a PolarPlex Kit (Millipore, Bedford, MA). Hybridization and detection reaction were carried out according to the PolarPlex protocol. A permanent image of the hybridization pattern and the VHb gene was obtained by exposing the membrane to an X-ray film.

In a typical autoradiogram obtained as a result of such an experiment, three VHb-CHO clones and the parental CHO-tPA are used. All VHb-CHO clones show two distinct bands hybridized to the VHb probe whereas the parental clone shows one band. The lower band is present in all four clones suggesting that this fragment of DNA exhibits a great deal of homology with the VHb gene and could be the endogenous hemoglobin gene found in Chinese hamster ovary cells. The upper band is present only in VHb-CHO cells indicating that this band corresponds to the VHb gene present in the vector pMSG-VHb that has been integrated in CHO cell chromosome as a result of transfection. Thus, the presence of VHb gene integrated into the chromosome of CHO-VHb cells has been established.

EXAMPLE 14 - OXYGEN-BINDING PROTEIN EXPRESSION IN CHO CLONES

Cell extracts were prepared for each sample as per the protocol described in Khosla et al. (2). Briefly, cells were harvested by trypsinization. The cell suspension was centrifuged at 2500 rpm for 10 minutes at 4°C. The resulting cell pellet was resuspended in 40 ml lysis buffer (100 Mm Tris pH 8.0, 10 mM NaCl and 10 mM EDTA pH 8.0). This cell suspension was subjected to freeze-thaw cycles 3 times in a dry ice-ethanol and 37°C waterbath for 5 minutes each. The resulting suspension was centrifuged at 12000 rpm for 2 minutes at 4°C. The supernatant was transferred to an Eppendorf tube and stored at -20°C until SDS-PAGE was performed.

SDS-PAGE was carried out using standard protocols using the Protein minigel apparatus (Biorad, Richmond, CA). The proteins were transferred to a nitrocellulose membrane and VHb detection was accomplished by Western analysis explained below. The membrane was incubated for 30 minutes with PBS containing non-fat dried milk to prevent non-specific binding. This was followed by a 30 minute incubation with rabbit anti-serum to VHb. The membrane was then subjected to two 10 minute washes with PBS. The membrane was then incubated with horseradish peroxidase conjugated to anti-rabbit antibody for 30 minutes. After two 10 minute washes with PBS, the membrane was then incubated with the horseradish peroxidase substrate 4-chloro-ß-naphthol for 30 minutes which resulted in appearance of bands corresponding to VHb protein. JM101:pRED2 (2) (this *E. coli* strain expresses high levels of VHb) cell extract was used as VHb standard.

EXAMPLE 15 - INDUCTION OF CLONED VHb EXPRESSION IN RECOMBINANT CHO CELLS

Cells expressing VHb were inoculated at 4 x 10^5 cells/dish. Twenty-four hours post in culation, dexamethasone (Sigma, St. Louis, MO) was added to each dish at final concentrations ranging from 0 to 2 μM. Cell growth was monitored everyday as described earlier. Cell extracts were prepared for Western blot analysis as described in the previous section. In one experiment, the VHb expression was monitored as a function of dexamethasone concentration (0.01, 0.5, 1.0, and 2.0 μM) after 50 hours of induction. In the other experiment, VHb expression was monitored for a single dexamethasone concentration for induction times of 24, 28, 72, and 96 hours induction period. Initial experiments were done with three VHb-CHO clones described in Example 14, namely, 30, 4 and 19. All three express VHb upon induction. Only results for VHb-CHO clone 30 are discussed below.

The results of Western blots from such experiments show that VHb expression is observed in these cells for all concentrations ranging from 0.1 to 2.0 μM. Moreover, VHb expression is observed in samples with induction times of 24 to 96 hours. The uninduced sample (no dexamethasone) also shows a very faint band corresponding to VHb indicating that the MMTV promoter is slightly leaky under conditions of the experiment. This data shows that these CHO cells express VHb upon induction.

EXAMPLE 16 - EFFECT OF CLONED VHb ON tPA PRODUCTION BY RECOMBINANT CHO CELLS

The clones used for this study were the parental CHO-tPA and the VHb-expressing CHO-tPA clone. For the VHb-expressing cells, dexamethasone concentrations of 0, 0.1 and 0.5 μM were used. These cells were

induced on day 1 of the batch culture. All experiments were carried out using 100 x 20 tissue culture dishes. Cells were inoculated on day 0 at a density of 4 x 10^5 cells per dish. On every day of the 5-day batch culture experiment, one dish was removed from the incubator for measurements of cell number, tPA titers and other supernatant metabolite concentrations. Total cell count was monitored using a Coulter Counter. Viability was measured by the trypan blue exclusion method using a hemocytometer. Supernatant was frozen at -20°C for tPA and metabolite analysis. The tPA production was monitored using an ELISA kit (COALIZA, KabiVitrum, Franklin, OH) according to the standard protocol provided by the manufacturer. tPA concentrations in each sample were calculated by using a calibration curve obtained using standard tPA samples provided in the kit.

The total amount of tPA produced each day was calculated by multiplying the concentration of tPA obtained by the volume of supernatant present in each dish. This was done to account for the progressive reduction in volume of the supernatant due to evaporation of water during the course of the batch culture. On each day, cell extracts were prepared and stored as described earlier for analysis of VHb expression during the batch culture. The day 1 sample from VHb-expressing cells corresponds to the uninduced level of VHb expression whereas the CHO-tPA sample serves as negative control for VHb expression since these cells lack the VHb gene. This experiment was carried out two times in order to establish reproducibility and consistency of our experiments. The results of such an experiment are discussed below.

Figures 4 A, B, C, and 5 A, B and C show the total cell number, total tPA produced and total tPA produced/10^6 cells as a functi n of batch culture time for th se two independent experiments. From both these experiments, it is clear that the specific growth rates of VHb-CHO cells is about 20-30% lower than that of the parental CHO-tPA clone. However, this effect is not due to VHb expression since the uninduced VHb-CHO cells show almost the same growth characteristics as the induced VHb-CHO cells. This effect is probably due to some unknown host-vector interaction that manifests itself as a result of integration of transfected DNA sequences into the host cell chromosomes. However, the tPA productivity characteristics are significantly different in the VHb-CHO clone as compared to the parental CHO-tPA clone. The total tPA amounts as well as the amount of tPA produced per cell are about 50-100% higher in VHb-CHO cells compared to these properties in the parental CHO-tPA cell culture.

References:

1. Maniatis, T., Fritsch, E.F. and Sambrook, J. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989.

2. Khosla, C. and Bailey, J.E. Mol. Gen Genet., 214:158-161 1988.

EXAMPLE 17 - EXPRESSION OF A BACTERIAL HEMOGLOBIN IN STREPTOMYCES ENHANCES CELL GROWTH AND OXYGEN UPTAKE RATES UNDER OXYGEN-LIMITED CONDITIONS.

A plasmid was constructed for the expression of a bacterial hemoglobin in Streptomyces. This plasmid, pWLD5, contains the Vitreoscilla hemoglobin gene and its native transcriptional regulatory sequences [Khosla and Bailey (1988) Mol. Gen. Genet., 214:158] cloned into a common Streptomyces plasmid, pIJ699 [Keiser and Melton (1988) Gene, 65:83]. Specifically

the 1.2 kilobase Hind III/SphI Vitreoscilla DNA fragment containing the hemoglobin gene was first inserted into the HindIII/SphI site of the E. coli plasmid pUC19. This construct was then linearized with Hindiii and ligated into HindIII-cut pIJ699. The resulting plasmid, pWLD5, was stably maintained in both E. coli and Streptomyces lividans.

To investigate the effect of hemoglobin on cell growth rate, S. lividans strain TK64 (pro2, str6, obtained from Dr. David Hopwood, John Innes Institute, Norwich, England) was transformed with pWLD5 DNA. A single thiostrepton-resistant colony, designated TK64:pWLD5, was selected for further experiments. Hemoglobin expression in TK464:pWLD5 was confirmed by Western analysis of total cell protein. A crude cell extract was generated by sonication and the proteins separated by SDS-polyacrylamide gel electrophoresis. The proteins were then electrotransferred to nitrocellulose membrane and screened with polyclonal antiserum generated against pure Vitreoscilla hemoglobin. A hemoglobin band of identical molecular weight as pure hemoglobin was detected in the cell extracts. Hemoglobin expression appeared to be constitutive as the levels were similar in cells sampled from any stage of growth. Expression of functional hemoglobin was demonstrated by a carbon monoxide difference spectrum technique [Webster and Liu (1974) J. Biol. Chem. 249:4257].

To investigate the effect of hemoglobin expression on cell growth and respiration, TK64:pWLD5 was compared with the plasmid-free strain (TK64) under two culture conditions corresponding to high and low aeration. The culture medium used for the experiment was as follows: 3% dextrose, 2% N-Z amine Type I, 1% yeast

extract, and 1% v/v trace elements mix (0.1% $FeSO_4 \cdot 7H_2O$, 0.1% $MnSO_4 \cdot 7H_2O$, 0.0025% $CuCl_2 \cdot 2H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.00056% H_3BO_3, 0.002% $ZnSO_4 \cdot 7H_2O$, 0.0019% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$). 5 ug/mL of thiostrepton was added to the TK64:pWLD5 culture. The first condition (high aeration) was a 50 mL culture volume in a 250 mL unbaffled erlenmeyer flask shaken at 250 rpm at 300C. The second condition (low aeration) was a 75 culture volume in a 250 mL unbaffled erlenmeyer flask shaken at 150 rpm at 30°C. With high aeration, the two strains had similar maximum specific growth rates (0.22-0.24 h^{-1}) but the plasmid-free strain reached a higher final cell density (OD_{590}=7.0) compared to TK64:pWLD5 (OD_{590}=5.0). With lower aeration, however, TK64:pWLD5 reached a higher final cell density (OD_{590}=1.95) than the plasmid-free strain (OD_{590}=1.25). This represents a 56% increase in the final cell density in cells expressing hemoglobin under reduced aeration conditions. The maximum specific growth rates of the two strains were similar (0.10-0.11 h_{-1}) under reduced aeration. Hemoglobin expression levels in the two strains were similar throughout the experiment as demonstrated by Western analysis.

Oxygen uptake rates (OUR's) were compared between TK64:pWLD5 and the plasmid-free strain throughout this experiment. Cells were removed at various times, washed, and resuspended in fresh medium at an OD_{590} of 0.10. The OUR's were then measured using a Yellow Springs instruments biological oxygen monitor. The rates were normalized to cell weights and compared throughout the growth curve (Table 1). Although the OUR's of the two strains were similar throughout the experiment with high aeration (Table 1A), they were consistently higher in the hemoglobin-expressing strain with lower aeration, especially at

the later stages of growth (Table 1B). For example, at an OD_{590} of approximately 0.6, the OUR for the plasmid-free strain was 0.22 mM O_2/h-g whereas the OUR for TK64:pWLD5 was 0.29 mM O_2/h-g, a difference of 32%.

This experiment indicates that Streptomyces lividans cells expressing a bacterial hemoglobin grow to significantly higher cell densities and have higher oxygen uptake rates than the non-expressing strain under reduced aeration conditions. A similar plasmid, pWLD15, containing the same Vitreoscilla hemoglobin gene (including its transcriptional regulatory sequence) fragment as that in pWLD5, except that it was cloned into the opposite orientation, also expresses hemoglobin in Streptomyces lividans. This latter finding is evidence that the expression of the hemoglobin gene originates in the inserted fragment (originating from Vitreoscilla) as opposed to elsewhere on the Streptomyces-based pIJ699 plasmid.

Table 1A - High aeration

Strain	$O.D._{590}$	O.U.R. (mM O_2/h-g)
TK64	0.5	0.32
TK64:pWLD5	0.4	0.35
TK64	0.9	0.32
TK64:pWLD5	0.8	0.33
TK64	5.0	0.11
TK64:pWLD5	4.6	0.12

Table 1B - Low aeration

Strain	$O.D._{590}$	O.U.R. (mM O_2/h-g)
TK64	0.3	0.35
TK64:pWLD5	0.3	0.42
TK64	0.6	0.22
TK64:pWLD5	0.6	0.29
TK64	2.0	<0.10
TK64:pWLD5	2.0	0.27

EXAMPLE 18 - GROWTH ENHANCEMENT OF HEMOGLOBIN-EXPRESSING STREPTOMYCES GROWN UNDER TWO ADDITIONAL CONDITIONS OF REDUCED OXYGEN.

The enhanced growth of hemoglobin-expressing Streptomyces was examined under two additional conditions of low aeration in shake flask cultures. Strains TK64 (no plasmid) and TK65:pWLD5 were cultured in 12.5 and 25 mL culture volumes in 250 mL flasks for 72 hours at 150 rpm at 30°C. The medium used was the same as in Example 17. The final cell densities were measured at OD_{590}. in the 12.5 mL culture, TK64:pWLD5 reached a final OD_{590} of 5.8 while TK64 reached an OD_{590} of only 4.0, a difference of 45%. In the 25 mL culture, TK64:pWLD5 reached a final OD_{590} of 4.5, while TK64 reached an OD_{590} of only 3.3, a difference of 41%. This experiment indicates that hemoglobin expression benefits Streptomyces cell growth under two additional conditions of reduced culture oxygen.

EXAMPLE 19 - EXPRESSION OF BACTERIAL HEMOGLOBIN IN STREPTOMYCES COELICOLOR.

To demonstrate that Vitreoscilla hemoglobin can be expressed in another streptomycete, a plasmid similar to pWLD5 was constructed by inserting BamHI-linearized pRED2 [Khosla and Bailey (1988) Mol. Gen. Genet. 214:158] into BgIII-digested p1J699. The plasmid pRED2 contains the identical hemoglobin sequence as pWLD5 but contains an additional 1.5 kb

of non-essential DNA. The resultant plasmid, pWLD10, was transformed into Streptomyces coelicolor strain M145 (SCP1-, SCP2- obtained from Dr. David Hopwood, J hn Innes Institute, Norwich, England) and a single thiostrepton-resistant transformant, designated M145:pWLD10, was selected for further experiments.

M145:PWLD10 cells were grown in liquid culture to exponential phase in 50 mL YEME medium (0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 1% glucose, 34% sucrose, 5 mM $MgCl_2 \cdot 6H_2O$) at 250 rpm at 30°C. A cell extract was prepared by sonication and the proteins separated by SDS-PAGE and screened with anti-Vitreoscilla hemoglobin antiserum. Western analysis indicated that a significant level of hemoglobin of identical molecular weight as pure Vitreoscilla hemoglobin was present in cell extracts of M145:pWLD10 but not in the plasmid-free strain. This indicates that Vitreoscilla hemoglobin is stably expressed in another species of Streptomyces.

These data also indicate that the Vitreoscilla hemoglobin promoter element functions in S. coelicolor to express a heterologous protein. Thus, this promoter functions in different strains of Streptomyces.

EXAMPLE 20 - EXPRESSION OF BACTERIAL HEMOGLOBIN IN STREPTOMYCES COELICOLOR RESULTS IN HIGHER FINAL ANTIBIOTIC LEVELS.

Antibiotic production in Streptomyces coelicolor strains M145 and M145:pWLD10 was compared in a shake flask culture experiment. One mL of exponential phase cells were inoculated into 50 mL of YEME medium (5 ug/ml thiostrepton was added to the M145:pWLD10 culture) in 250 mL unbaffled flasks. The cells were grown at 250 rpm at 30°C. Ten days later the cultures were analysed for the production of the

pigmented antibiotic, undecylprodigiosin. The assay was performed by mixing equal volumes of the culture and 0.1 M NaOH followed by a 30" sonication (50 Watt output) on ice. The sonicate was then filtered through a 0.2 uM nitrocellulose membrane. The OD_{468} of the filtrate, which is a measure of undecylprodigiosin, was then determined. While the hemoglobin-expressing strain had an OD_{468} of 1.4, the non-expressing strain had an OD_{468} of only 0.6. This indicated that greater than twice as much antibiotic is produced in a hemoglobin-expressing strain of Streptomyces.

EXAMPLE 21 - EXPRESSION OF BACTERIAL HEMOGLOBIN IN CORYNEBACTERIA

A plasmid was constructed for the expression of a bacterial hemoglobin in Corynebacteria. This plasmid, pBHb3, contains the Vitreoscilla hemoglobin gene (Khosla and Bailey, Mol. Gen. Genet., 214:158, 1988) cloned into a common Corynebacterium plasmid pBK10. Specifically, a 5.5 kilobase plasmid pINT1 (Khosla and Bailey, J. Mol. Biol., 210:79, 1989) which consists of the E. coli plasmid pBR322 with a 1.2 kilobase insert consisting of the Vitreoscilla hemoglobin gene and the 122 base pair tac promoter (P.L. Biochemicals), was digested with Sal 1 and EcoR1. The plasmid ends were then made blunt by filling in with DNA polymerase 1 (Klenow fragment), and the 1.5 kilobase fragment containing the Vitreoscilla hemoglobin gene, tac promoter and flanking pBR322 sequences was isolated. This fragment was ligated to EcoR1 linearized plasmid pBK10 (Paradis, et al., Gene, 61:199, 1987), the ends of which had also been made blunt. The resulting fragment was made circular with T4 DNA ligase. This plasmid, pBHb3, was transformed into E. coli, and was stably maintained by selection with the antibiotic

kanamycin. Corynebacterium glutamicum strain ATTC 39022, a variant of the wild type C. glutamicum strain ATTC 13032, was transformed with pBHb3 DNA out of E. coli. A singl kanamycin resistant colony was isolated. This clone was designated 39022:pBHb3-7. The wild-type C. glutamicum strain ATTC 13032 was then transformed with pBHb3 DNA isolated out of clone 39022:pBHb3-7 and a kanamycin resistant colony, designated 13032:pBHb3 15 was selected for further experiments. Hemoglobin expression in 13032:pBHb3-15 was confirmed by Western analysis of total cell protein. A crude cell extract was generated by sonication and the proteins separated by SDS-polyacrylamide gel electrophoresis. The proteins were then electrotransferred to a nitrocellulose membrane and screened with polyclonal antiserum generated against Vitreoscilla hemoglobin purified from E. coli, harboring plasmid pRED2 (Khosla and Bailey, Mol. Gen. Genet., 1988). A band of identical molecular weight as pure hemoglobin was detected in the cell extracts.

EXAMPLE 22 - EXPRESSION OF A BACTERIAL HEMOGLOBIN IN CORYNEFORMS ENHANCES AMINO ACID YIELD AND PRODUCTIVITY IN SHAKE FLASK CULTURES

Lysine production in Corynebacterium glutamicum ATCC 13287 and in similar cells transformed with the plasmid pBHb3 was compared in a shake flask culture experiment. Equal amounts of exponential phase cells were inoculated into 75 mL (250 mL flasks) of the following medium: glucose, 175 g/L; yeast extract, 2 g/L; ammonium sulphate, 55 g/L; magnesium sulfate (heptahydrate), 0.8 g/L; potassium phosphate, 1 g/L; manganese sulfate (tetrahydrate), 0.01 g/L; ferrous sulfate (heptahydrate), 0.01 g/L; biotin, 100 mg/L; thiamine-HCl, 200 mg/L; L-leucine, L-methionine and L-threonine, 0.001 mM each. The pH of the culture

was maintained at neutrality by adding 50 g/L of calcium carbonate to each flask. The cells were grown at 250 rpm shaking speed and 30°C. Samples were taken at different times, and the culture optical density was measured at 600 nm in a spectrophotometer. An OD 600 of 1.0 was determined to correspond to a dry cell weight of 0.35g/Liter. Glucose and lysine concentrations in the sample supernatants were measured by high performance liquid chromatography. Cell extracts were prepared from samples taken from each time point throughout the experiment. Proteins were separated by SDS-PAGE and screened with antiserum against Vitreoscilla hemoglobin. Western analysis confirmed that hemoglobin was being expressed throughout the experiment. This hemoglobin was demonstrated to be functional by a carbon monoxide difference spectrum technique (Webster and Liu, J. Biol. Chem., 1974).

Table 2-1 shows the results obtained 48 hours after inoculation.

Table 2-1: Effect of hemoglobin expression on the production of L-lysine in C. glutamicum ATCC 13287

Strain	13287:pBHb3	13287
glucose (g/L)	159	155
lysine (g/L)	1.60	1.45
OD (600 nm)	5.14	5.67
cell mass (gdw/L)	1.8	2.0

Although the cell yield per glucose consumed is similar in both strains (110 g/kg vs 100 g/kg), the lysine yield per glucose consumed is 43% higher in the hemoglobin containing cells (100 g/kg va 0.07 g/kg). Also, the lysine produced per cell mass is

25% higher in th hemoglobin containing cells (910 g/kg vs 730 g/kg).

Yields:		
g cells/kg glucose	110	100
g lysine/kg glucose	100	70
g lysine/kg cells	910	730
Productivity:		
g lysine/(kg cells.h)	19	15

This experiment indicates that hemoglobin expression in Coryneform increases lysine yield and productivity.

EXAMPLE 37 - GROWTH ENHANCEMENT OF E. COLI IS DUE TO THE OXYGEN BINDING PROPERTIES OF VITREOSCILLA HEMOGLOBIN

In this experiment we compare the growth properties in shake flasks of the recombinant strains expressing Vitreoscilla hemoglobin (LE392:pINT1) and a truncated version (LE392:pBST) of the E. coli hemoglobin-like protein described in example yy with similar plasmid-containing (LE392:pUC18) and plasmid free (LE392) cells. LE392:pBST cells give a negative CO-binding spectrum indicating that the truncated E. coli hemoglobin protein is not biologically active. Experimental medium and conditions were as described in example 36.

Results:
Cell growth as measured by culture optical density after 24th of inoculation is listed in the following table and graphically in FIG. 7:

	LE392	LE392: pUC18	LE392: pINT1	LE392: pBST
Stationary phase				
cell density	5.00	5.32	8.76	5.24
OD (600 nm)				

Conclusion:

Cells containing a biologically active hemoglobin (LE392:pINT) grow to higher cell densities than cell containing a hemoglobin that does not bind oxygen and thus is biologically inactive (LE392:pBST). Cells expressing inactive hemoglobin reach cell densities similar to control cells containing the parent plasmid pUC18 and to no-plasmid control cells.

EXAMPLE 23 - THE PRESENCE OF ACTIVE VITREOSCILLA HEMOGLOBIN IN CORYNEBACTERIUM GLUTAMICUM ENHANCES LYSINE PRODUCTION, YIELD, AND OXYGEN UPTAKE RATE IN BATCH FERMENTATION

In this example, lysine production by C. glutamicum ATCC 13287 cells transformed with the plasmid pBHb3 and plasmid-free cells was studied in batch fermentation experiments.

C. glutamicum ATCC 13287:pBHb3 and C. glutamicum ATCC 13287:no-plasmid cells were grown in 250-mL shake flasks at 30°C and 250 rpm in the following synthetic medium: glucose, 75 g/L; yeast extract, 2 g/L; ammonium sulfate, 55 g/L; magnesium sulfate (heptahydrate), 0.8 g/L; potassium phosphate, 1 g/L; 2manganese sulfate (tetrahydrate), 0.01 g/L; ferrous sulfate (heptahydrate), 0.01 g/L; biotin, 100 mg/L; thiamine-HCl, 200 mg/L; L-leucine, L-methionine and

L-threonine, 200 mg/L ach; p 7.0. These cells were used to seed the ferment rs.

Fermentations were conducted in 3-L B. Braum MD fermentors in the medium described above, at 30°C and under constant air sparging (0.5 L/min). Initially, the impeller agitation rate was constant. During that time, the dissolved oxygen concentration gradually decreased. When the dissolved oxygen concentration reached 5% of air saturation, the agitation rate was adjusted by the fermentor controller in order to maintain the dissolved oxygen concentration at 5% of air saturation until the end of the fermentations. The culture pH was maintained at pH 7.0 by the fermentor pH controller by periodic additions of 4 N sodium hydroxide.

Samples were taken at different times throughout the fermentations. Glucose and lysine concentrations were determined HPLC. Optical density was measured at 600 nm using a Beckman spectrometer. An OD600 of 1.0 corresponds to a cell mass of 0.35 g dw/L. Analysis of the off-gas oxygen and carbon dioxide content were done using a Perkin-Elmer MGA 1200 mass spectrometer. Oxygen uptake rates (OUR) and respiration coefficients were calculated using the off-gas data. Hemoglobin expression was stable throughout the fermentation as demonstrated by Western electroblotting of the samples taken. The hemoglobin produced by 13287:pBHb3 was biologically active as demonstrated by carbon monoxide binding assay.

Table 3.1 shows the optical density, glucose and lysine concentration as a function of time for 13287:pBHb3 and 13287:np.

Table 3.1

Time (h)		OD600	glucose (g/L)	lysine (g/L)
10	13287:pBHb3	3.5	64	0.1
	13287:np	3.7	74	0.1
20	13287:pBHb3	23	47	4.4
	13287:np	24	47	4.6
35	13287:pBHb3	35	20	15
	13287:np	36	27	12
45	13287:pBHb3	44	5	22
	13287:np	44	11	16
56	13287:pBHb3	42	0.1	27
	13287:np	43	0.2	21

The cell yield per glucose consumed is similar in both strains: 196 g cells/kg glucose for 13287:pBHb3 and 201 g cells/kg glucose for 13287:np. The lysine yield per glucose consumed is 360 g/kg glucose for 13287:pBHb3 and 289 g/kg for 13287:np. The hemoglobin containing cells have a lysine yield 25% higher than the no-plasmid cells. Also, the lysine produced per cell mass is 31% higher in the hemoglobin-producing cells (1,840 g/kg cells for 13287:pBhb3 vs 1,400 g/kg cells for 13287:np). Accordingly, lysine productivity per cell mass is 31% higher in the hemoglobin containing cells (32.9 g/kg cells/h for 13287:pBHb3 and 25.0 g/kg cells/h for 13287:np).

Table 3.2 shows the results from the off-gas analysis during the lysine production period for the 13287:pBHb3 and 13287:np fermentations.

Table 3.2:

Time (h)		OUR (mmol/L/h)	PQ
35	13287:pBHb3	11.2	0.98
	13287:np	7.60	1.05
45	13287:pBHb3	10.2	1.06
	13287:np	6.40	0.92
56	13287:pBHb3	11.1	1.06
	13287:np	6.60	0.94

During the lysine production phase (35, 45 and 58 h), the oxygen uptake rate (OUR) of 13287:pBHb3 averages 10.8 mmol/L/h, a 57% higher than the average OUR of 13287:np during the same time period (6.87 mmol/L/h).

These results indicate that expression of active hemoglobin in Coryneforms increases lysine yield and productivity, and oxygen uptake rate.

EXAMPLE 24 - CONSTRUCTION OF A TRANSFORMATION VECTOR FOR FILAMENTOUS FUNGI

Plasmid pENT 10 is a vector which contains the VHb gene driven by the strong fungal promoter TR-1 isolated from *Trichoderma reesei*. Selection in fungi is by the Sh *ble* gene product, isolated from *Streptoalloteichus hindustanus*, which confers phleomycin resistance to the host. This gene is driven by the fungal promoter GPD isolated from *Aspergillus nidulans*. For bacterial manipulations, selection is tetracycline resistance.

Construction of vector:

A plasmid which confers Tet resistance (Tet r) was constructed first as a base for manipulation in E. coli. Tet r versus Amp r was chosen for an integration plasmid for *Penicillium* since the Beta-lactamase product from the Amp r gene is destructive to the penicillin product and hence would interfere with penicillin production results.

The Tet r vector was constructed by isolating the Tet r gene from a pBR322 based vector and cloning it into pUC 18 (Yanisch-Perron et al., Gene 33:103 (1985)) so as to disrupt the Amp r gene. The tet r gene carried on pVU-2 on an AvaI fragment was inserted via blunt ends into ScaI-AvaII sites of

pUC 18, which inactivtes the amp r g ne. The resulting vector was nam d pTAS 18R.

The cloning of the VHb gene was acc mplished by isolating the VHb gene from plasmid p TacHb (Example 5) via a Xba I-Sph I fragment. The TR-1 promoter fragment was isolated from plasmid pUT 737 (purchased from CAYLA, France) on a Nde I-Spe I fragment. These DNA fragments were ligated to vector pTAS 18R cut with Nde I-Sph I. The resulting intermediate vector was named pENT 1B. The Sh ble gene, GPD promoter and Trp C terminator region were isolated from vector pUT 720 (CAYLA, France) by a Eco RI-Xba I digest, Klenowed, and inserted into the Bam HI site of pENT 1B which had also been treated with Klenow to remove protruding DNA ends and create a blunt end cloning site. The resulting vector was named pENT 10.

EXAMPLE 25 - TRANSFORMATION OF PENICILLIUM CHRYSOGENUM

Transformation into fungi is accomplished by breaking down cell walls via enzymatic digestion to form protoplasts which are then selectively permeable to uptake of exogenous DNA. DNA which is transformed into fungi is not maintained as plasmid DNA as in *E. coli* but integrated into the host genome.

Buffers used in procedure

KMC (osmotic stabilization buffer)
700mM KC1
50mMC CaC12
10 mM MES (buffering agent)
-pH is brought to 5.8 by addition of HCl

Lytic Buffer
50mM Phosphate buffer pH 5.8
700mM KC1

PMC (Aids in uptake of DNA)
50% PEG 8000
50 mM CaC12
10 mM MES
-pH is brought to 5.8 by addition of HCl

NaCl (osmotically stable wash)
0.9% soln

Basic Proc dure for transformation:

- inoculate conidia in minimal media (200 ml), supplemented with 2.5 mM CaCl2 and 200µl of a standard trace element solution.

- grow at 28°C for 40 hours.

- mycelia are recovered by filtration through a 30µm filter and washed with a 0.9% solution.

- Protoplasting and transformation procedures were carried out essentially as those described in Cantoral J.M. et al, Bio/Technology, Vol 5, May 1987 pg.494-496.

- Selection of protoplasts was on minimal osmotic media supplemented with 0.5% yeast extract containing 20µg/ml of phleomycin (CAYLA, France) at 28°C and overlayed with the same media in a soft agar base after 24 hours.

EXAMPLE 27 - EXPRESSION OF A BACTERIAL HEMOGLOBIN IN *PENICILLIUM CHRYSOGENUM* IMPROVES FINAL ANTIBIOTIC LEVELS.

In this example, p nicillin production was studied in hemoglobin-expressing *P.chrysogenum* and in non-expressing control cells in batch fermentations. The plasmid pENT10 described in Example 24 was used for integration of the *Vitreoscilla* hemoglobin gene into the genome of *Penicillium chrysogenum* ATCC 48271.

Medium and conditions: The seed medium was as follows (per liter): 30 g glucose, 10 g lactose monohydrate, 30 ml corn steep liquor, 2 g ammonium sulfate, 5 g calcium carbonate, 0.5 g potassium dihydrogen phosphate, 10 g Pharmamedia, 10 g yeast extract. Two hundred ml of the seed medium was inoculated with spores to a final concentration of 1×10^8 spores/ml. The seed cultures were grown at 30°C for 48 hours at 220 rpm and were used to inoculate 2-liter fermentors. The fermentation medium consisted of the following (per liter): 120 g lactose monohydrate, 27.5 g Pharmamedia, 10 g ammonium sulfate, 10 g calcium carbonate, 10 g lard oil, 0.5 g fermentation cultures were grown at 30°C for 24 hours in B. Braun MD fermentors. The air flow rate was maintained at 1 l/min and the dissolved oxygen was controlled at 30% of air saturation throughout the fermentation. At 24 hours, the temperature was reduced to 25°C, keeping all other conditions the same, and a phenylacetic acid (potassium salt) feed was initiated at a rate of 0.07 g/h. Throughout the duration of the fermentation, the off-gas was analyzed by mass spectrometry (model 1200, Perkin-Elmer, USA).

High pressure liquid chromatography (HPLC) was employed to determine the species and concentrations of penicillin present in the fermentation broths.

Samples for HPLC analysis were removed from the fermentors, filter d through sterile 0.2 um filters and frozen at -20°C or assayed immediately. The filtrate penicillin concentrations were measured using a Shimadzu HPLC system. Penicillin G obtained from Sigma was used as the standard. Samples, after dilution with buffer A, were analyzed for penicillin using an AXXI-CHROM C-18 (ODS) column (Cole Scientific, Calabasas, CA). Buffer A was a 0.015 M solution of ammonium acetate in 5% (v/v) methanol. Dilutions of the samples and the standard preparations were made with this buffer. Buffer B was a 0.015 M solution of ammonium acetate in 30% (v/v) methanol. The peaks were eluted over a binary gradient and were detected at 220 nm in a UV spectrophotometer (model SPD-6AV, Shimadzu, Japan). Peak areas were calculated by an integrator (model CR501, Shimadzu, Japan). The levels of penicillin concentrations obtained in shake flask cultures were elevated by greater than 20% in strains transformed with the VHb gene relative to control strains.

EXAMPLE 28 - CLONING OF HMP PROTEIN IN E. coli AND TRUNCATED hmp GENES

A plasmid was constructed for the purpose of enhancing the expression of the hmp gene (hemoprotein) in E. coli. The hmp gene (S.G. Vasudevan, et al. Mol Gen Genet, 1991, vol, 226: 49-58) encodes a 44 kDa protein which has dihydropteridine reductase (DHPR). The hmp amino acid sequence bears great similarity to that of the Vitreoscilla hemoglobin (VHb) gene having 46% identity through the first 146 amino acids.

Two synthetic oligomers were prepared based upon the dmp nucleotide sequence as published by S.G. Vasudevan. A 25-oligomer encodes th first 18

nucleotides of the hmp gene starting from the first ATG, preceded by a synthetic restriction enzyme site (Xbal) and consists of the sequence 5' CTC-TAG-AAT-GCT-TGA-CGC-TCA-AAC-C 3'. The second 25-obigomer encodes the complement to the end of the hmp gene starting with the first stop codon, preceded by a synthetic restriction enzyme site (Kpn 1) and consists of the sequence 5' AGG-TAC-CTT-ACA-GCA-CCT-TAT-GCG-A 3'. These two oligomers were used to perform a PCR reaction in which the template was genomic DNA isolated from E. coli strain K-12. A 1200bp PCR product was generated which restriction analysis showed to be the hmp gene. This PCR product was digested with Xba 1 and Kpn 1, and ligated to the plasmid pTacVhb from which the VHb sequence has been removed by digestion with Xba 1 and Kpn 1. The ligation mix was used to transform competent E. coli ceells and transformants were selected by resistance to the antibiotic amplicilin. Several resistant clones were geneerated from which plasmid DNA was isolated. Restriction analysis showed one of the plasmids to be correct and it waS designatted pTac-HMP. The expression of pTac-HMP in E. coli was confirmed by a carbon monoxide difference spectrum technique (Webster and Liu, J. Biol. Chem., 1974). The difference spectrum of control cells not transformed showed no Co-binding activity, while cells transformed with pTac-HMP showed the difference spectrum characteristic of cells expressing functional hemoglobin and hemoglobin-like (hmp) proteins.

Two additional plasmids were constructed which are identical to pTac-HMP through that portion of the hmp sequence which is similar to the VHb sequence, but which have the dis-similar hmp sequences removed. Plasmid pTac-Bst was constructed by digesting

pTac-HMP with Xba 1 and Mlu 1. An approx. 400bp fragment was isolated which consists of the first portion of the hmp gene from the first ATG to a unique Mlu 1 site, and encodes the first 118 amino acids of the hmp protein. This fragment was ligated into plasmid pTac-VHb which had been cut with Xba 1 and Mlu1 in-order to remove the first 120 amino acids of the VHb gene. Plasmid pTac-Bst thus contains a DNA sequence which encodes the first 118 amino acids of the hmp gene followed by the last 26 amino acids of the VHb gene. These two segments are joined at a unique Mlu 1 site which both genes have in-common. Plasmid pTac-Bst was transformed into E. coli cells and expression was confirmed by Western analysis. A crude cell extract was generated by sonication and the proteins separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane and screened with polyclonal antiserum generated against Vitreoscilla hemoglobin. A band of identical molecular weight as pure hemoglobin was detected in the cell extract. A carbon monoxide difference spectrum was performed, however no functional protein could be detected by this method.

The third plasmid was constructed by using PCR to generate a truncated version of the hmp gene and then ligating the product into pTacVHb from which the VHb sequences had been removed. Specifically, a 23-oligomer was designed which encodes the complement to 17 nucleotides of the hmp DNA sequence from a region aprox. 7 amino acids past the end of the portion of the hmp gene which is similar to the VHb gene. These 17 nucleotides are preceded by an additional 6 nucleotides which encode the restriction enzyme site Bsu36I. The Bs36 I site was designed to encode a stop codon. This 23-oligomer consists of the

sequence 5' ACC-TTA-GGC-TTT-GCT-GGC-GTT-TT 3'. A PCR reaction was carried out using this 23-oligomer and the 25-oligomer which codes for the first porti n of the hmp gene, and using plasmid pTac-HMP DNA for template. A PCR product of aprox. 440bp was generated which restriction analysis showed to be a portion of the hmp gene. The 3' end of this product was digested with the restriction enzyme Bsu36 I and made blunt by filling in with DNA polymerase I (Klenow fragment). This DNA fragment was then cut with the restriction enzyme Xba 1 and ligated into plasmid pTacVHb which had been cut with EcoR1, been made blunt-ended by filling-in with Klenow, and then digested with Xba 1 to remove the VHb sequence. The ligation mix was transformed into competent E. coli cells and a transformant which contained a correct plasmid construct as judged by restriction analysis was isolated. This plasmid was designated pTac-K12Hb. The E. coli clone transformed with pTac-K12Hb was used for two experiments. Western analysis was performed to confirm the expression of the truncated hmp gene, however no hybridization to Vitreoscilla hemoglobin anti-sera could be detected. A carbon monoxide difference spectrum was performed, but no functional hmp protein could be detected.

EXAMPLE 29 - CONSTRUCTION OF TRUNCATED VERSIONS OF E. COLI hmp AND VECTORS FOR EXPRESSION IN E. COLI

Two plasmids were constructed which are identical to pTac-HMP through that portion of the hmp sequence which is similar to the VHb sequence, but which have the dis-similar hmp sequences removed. Plasmid pTac-Bst was constructed by digesting pTac-HMP with Xba 1 and Mlu 1. An aprox. 400bp fragment was isolated which consists of the first portion of the hmp gene from the first ATG to a unique Mlu 1 site, and

encodes the first 118 amino acids of the hmp protein. This fragment was ligated into plasmid pTacVHb which had been cut with Xba 1 and Mlu 1 in order to remove the first 120 amino acids of the VHb gene. Plasmid pTac-Bst thus contains a DNA sequence which encodes the first 118 amino acids of the hmp gene followed by the last 26 amino acids of the VHb gene. These two segments are joined at a unique Mlu 1 site which both genes have in-common. Plasmid pTac-Bst was transformed into E. coli cells and expression was confirmed by Western analysis. A crude cell extract was generated by sonication and the proteins separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane and screened with polyclonal antiserum generated against Vitreoscilla hemoglobin. A band of identical molecular weight as pure hemoglobin was detected in the cell extract. A carbon monoxide difference spectrum was performed, however no functional protein could be detected by this method.

The third plasmid was constructed by using PCR to generate a truncated version of the hmp gene and then ligating the product into pTacVHb from which the VHb sequences had been removed. Specifically, a 23-oligomer was designed which encodes the complement to 17 nucleotides of the hmp DNA sequence from a region aprox. 7 amino acids past the end of the portion of the hmp gene which is similar to the VHb gene. The 17 nucleotides are preceded by an additional 6 nucleotides which encode the restriction enzyme site Bsu36I. The Bsu36 I site was designed to encode a stop codon. This 23-oligomer consists of the sequence 5' ACC-TTA-GGC-TTT-GCT-GGC-GTT-TT 3'. A PCR reaction was carried out using this 23-oligomer and the 25-oligomer which codes for the first portion of the hmp gene, and using plasmid pTac-HMP DNA for

template. A PCR product of aprox. 440bp was generat d which restriction analysis showed to be a p rtion of the hmp gene. The 3' end of this product was digested with the restriction enzyme Bsu36 I and made blunt by filling in with DNA polymerase I (Klenow fragment). This DNA fragment was then cut with the restriction enzyme Xba 1 and ligated into plasmid pTacVHb which had been cut with EcoR1, been made blunt-ended by filling-in with Klenow, and then digested with Xba 1 to remove the VHb sequence. The ligation mix was transformed into competent E. coli cells and a transformant which contained a correct plasmid construct as judged by restriction analysis was isolated. This plasmid was designated pTac-K12Hb. The E. coli clone transformed with pTac-K12Hb was used for two experiments. A carbon monoxide difference spectrum showed no detectable functional activity for the truncated hmp protein.

EXAMPLE 29 CONSTRUCTION OF VECTOR FOR EXPRESSION OF HORSE-HEART MYOGLOBIN IN C. glutamicum

A 7.3Kb plasmid was constructed for the expression of horse Myoglobin in Coryneform bacteria. Plasmid pGYM was digested with restriction enzymes Xba1 and SSP1. The larger of two fragments was gel isolated. This fragment contained all of the myoglobin gene and some pUC sequence but no promoter. This fragment was ligated to a fragment of plasmid pTac-Bst which had been first digested with Mlu1, made blunt with DNA polymerase 1 (Klenow fragment), then digested with Xba1, the smaller of two fragments having been gel isolated. This smaller fragment consists of the Tac promoter and some pUC sequence. The two fragments were ligated together and transformed into competent E. coli cells. Transformants were screened for plasmid DNA consisting of the pUC vector having a

Tac-Myoglobin insert. One such transf rmant was found and its plasmid DNA, pUC-TacMyo, was isolated for further manipulations. Plasmid pUC-TacMyo was digested with restriction enzyme Hind III. This liberated an 800bp fragment consisting of the myoglobin gene preceded by the Tac promoter. This fragment was gel isolated, made blunt using Klenow, and ligated with a deleted version of plasmid pFS1 (pFS1 del). Plasmid pFS1 del had been prepared by first digesting Nhe1 to remove the Tac-VHb sequence, then filling in the ends with Klenow. The pFS1 del - TacMyo. ligation mix was transformed into competent Coryneform strain ATTC 13287. One Coryneform transformant showed a reddish pigment and plasmid isolated from this transformant was determined to be a correct construction by restriction analysis and in particular by the presence of a unique EcoR1 site which is present in the myoglobin sequence but is not present in the VHb sequence. This Coryneform transformant was designated 13287:pFS-TacMyo. The expression of functional myoglobin in 13287:pFS-TacMyo was demonstrated by a carbon monoxide difference spectrum.

EXAMPLE 30 - CONSTRUCTION OF VECTOR FOR THE EXPRESSION OF SOYBEAN LEGHEMOGLOBIN IS E. coli

A 5.2Kb plasmid was constructed for the expression of a soybean leghemoglobin protein (LHbc1) in E. coli. This plasmid, pKK-LHbc1, contains the cDNA to soybean leghemoglobin c1 cloned into the expression vector pKK 233-2 (Pharmacia). Specifically, a 3.5Kb plasmid pCD1 which consists of the E. coli plasmid pBluescript SK (Stratagene) with the 600bp insert consisting of a cDNA sequence to the soybean leghemoglobin c1 gene, was used as template in a polymerase chain reaction (PCR). In order to perform

the PCR two DNA oligomers were designed which were c mplementary to the 5' and the 3' ends of the leghemoglobin cDNA sequence. Each oligomer also included an additional 6 bases which code for a unique restriction site. The oligomer which is complementary to the 5' end of the + strand of leghemoglobin c1 cDNA starts with a Nco1 restriction site and consists of the sequence 5'-tcc-atg-ggt-gct-ttc-act-gat-aa-3'. The oligomer which is complementary to the 3' end of the - strand of leghemoglobin c1 cDNA starts with a Hind III site and consists of the sequence 5'-caa-gct-ttt-ttt-ttt-ttt-ttt-ttt-t-3'. A polymerase chain reaction amplified the 600bp LHb c1 sequence resulting in a PCR product having a Nco1 site at the 5' end and a Hind III site at the 3' end. This PCR product was ligated with the cloning plasmid pCR 1000 (Invitrogen). This ligation mix was transformed into competent E. coli cells and transformants were selected by resistance to the antibiotic kanamycin. Plasmid DNA was isolated from several transformants and screened for the presence of full-length LHb c1 cDNA sequence by restriction analysis and gel electrophoresis. One clone was found to be correct and was designated plasmid pTA-LHbc1. Plasmid pTA-LHbc1 was linearized with Spe1 which cuts 40bp past the 3' terminus of the LHbc1 insert. The plasmid ends were then made blunt by filling in with DNA polymerase 1 (Klenow fragment). The LHbc1 sequence was removed from the pCR 1000 vector by digestion with Nco1, and this 600bp fragment was purified by agarose gel electrophoresis. An expression vector, pKK 233-2 (Pharmacia) was prepared by first linearizing with Hind III and filling in the ends with DNA polymerase 1 (Klenow fragment), then digesting with Nco1. The purified 600bp LHbc1 fragment and the linearized pKK 233-2 were ligated together and transformed into competent

E. coli cells. Transformants were selected by resistance to the antibiotic ampicillin. Plasmid DNA was isolated from several transformants and screened for the pres nce of full-length LHbc1 sequence by restriction analysis and gel electrophoresis. One clone was found to be correct and designated clone pKK-LHbc1. Plasmid pKK-LHbc1 was transformed into E. coli strains JM101 and LE 392. A single ampicillin resistant transformant of each strain was isolated and designated JM101: pKK-LHbc1, and LE 392: pKK-LHbc1. Expression of functional leghemoglobin was confirmed in both transformants by carbon monoxide binding assay (Webster, D.A., 1974, J. Biol. Chem. 249:4257-4260). A crude cell extract was prepared from each transformant as well as control cells by sonication. Cell debris was removed by centrifugation and each extract was divided into two aliquots. One aliquot from each sample was left exposed to air while the second aliquot was saturated with carbon monoxide. After thirty minutes all air-exposed and carbon monoxide-saturated aliquots were reduced by the addition of dithionite. The absorbance spectra from 400 to 500 nm was measured for each aliquot. For each sample the difference in aliquots was plotted. Cell extracts of JM101: pKK-LHbc1 and LE 392: pKK-LHbc1 showed a difference in absorbance between the air-exposed and carbon monoxide-saturated aliquots at aprox. 417nm which indicates the presence of functional leghemoglobin protein.

EXAMPLE 31 - EXPRESSION OF THE OXYGEN BINDING PROTEINS MYOGLOBIN, LEGHEMOGLOBIN, AND HAEMOPROTEIN ENHANCES CELL DENSITY IN E. COLI SHAKE FLASK CULTURES

Cell densities achieved by E. coli strain LE 392 and LE 392 transformed with plasmids pINT1, pGYM (see example D), pTac-HMP, and pKK-LHbc1 was compared in a shake flask culture experiment. Equal amounts of exponential phase cells were inoculated into 12.5 ml (250ml flasks) of the following medium: 10g/L yeast extract, 0.5g/L KH2PO4, 1.5g/L K2HPO4, 4g/l (NH4)2SO4, 5g/L glucose, 1.0ml/L 1M MgSO4 7H2O, 0.0.5 ml 100mM MgC12 2H2O, and 0.2ml 100mM FeC13. The medium was supplemented with 1.0ml/L of the following trace metal solution: in 100mls 2.7g FeC13 6H2O, 0.2g ZnCl, 0.2g CaC12 2H2O, 0.2g NaMoO4 (VI), 0.19g CuSO4 5H2O, and 0.05 g boric acid. The medium was further supplemented with 1.0ml of the following vitamin solution: in 500mls 0.21g riboflavin, 2.7g pantothenic acid, 3.0g niacin/nicotanic acid, 0.7g pyridoxine, 0.03g biotin, and 0.02g folic acid. The pH of the medium was adjusted to pH7.3 and maintained with 12.1g/L Trisma base. The cells were grown at 350rpm shaking speed and 37 deg. C. Cell density was monitored by measuring absorbance at 600nm in a spectrophotometer (OD600). Cell density was monitored at hourly intervals from 19 to 23 hours after inocultion. Maximum cell density was determined to be when OD600 did not increase within one hour. The maximum cell density obtained for the parent E. coli strain LE 392 and four transformants is shown in Table C-1.

Table C-1: Effect of the expression of four oxygen-binding proteins on maximum cell density attained by E. coli strain LE 392.

Strain	Protein Product	Final OD600	% of LE 392
LE 392		9.2	100.0
LE 392: pINT1	Vitreoscilla hemoglobin	12.2	132.6
LE 392: pGYM	Horse heart myoglobin	12.3	133.7
LE 392: pTacHMP	E. coli haemoprotein	9.8	106.5
LE 392:pKK-LHbc1	Soybean leghemoglobin	10.7	116.3

After termination of the shake flask experiment cell extracts were prepared from each of the five strains and assayed for expression of functional oxygen-binding activity. Oxygen-binding activity in the four transformants was confirmed by the carbon monoxide difference spectrum technique of Webster (1974, J. Biol. Chem. 249:4257-4260). As can be seen, all the globin-containing strains outgrew the control strain. These proteins are diverse in nature, being from animal, plant, and microbial origin. Because the common function of all the proteins is to reversibly bind oxygen, it is probable that oxygen-binding is the primary property of these proteins that results in growth improvement.

EXAMPLE 32 - CONSTRUCTION OF PLASMID FOR EXPRESSION OF MYOGLOBIN

A plasmid used for the expression of myoglobin in Escherichia coli was constructed in the following manner. The structural gene encoding horse heart myoglobin was synthesized based on its amino acid sequence [Eur. J. Biochem. 11, 267-277, 1969]. The purified gene was then inserted into the E.coli pEMBL18+ [Methods Enzymol. 155, 111-119, 1987]. The myoglobin gene is under the control of the tac promoter. The resulting plasmid, called pGYM, was

transformed into E. coli strain LE392 (supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1).

The presence of functional myoglobin in LE392:pGYM cells was confirmed by two methods. First, LE392:pGYM cells were significantly darker than LE392 cells without plasmid or containing a plasmid without the myoglobin gene. This dark color is due to the high intracellular concentrations of heme which is bound to the myoglobin protein. Second, carbon monoxide difference spectral analysis indicated the presence of an abundant intracellular CO-binding protein in LE392:pGYM cells but not in the control cells. Thus, functional myoglobin has been demonstated to be expressed in E coli cells using the recombinant plasmid pGYM.

EXAMPLE 33 - EXPRESSION OF MYOGLOBIN IN *E.COLI*

The aim of this study was to show that expression of the oxygen-binding protein myoglobin in E. coli provides a growth benefit to cells as has been documented for Vitreoscilla hemoglobin (VHb) [Nature 331, 633-635, 1988, Patent # 5,049,493]. The following strains were tested in shake-flask cultures:

Strain	Comment
LE392	Host strain (without plasmid)
LE392:pUC18	Plasmid control strain
LE392:pGYM	Myoglobin-expressing strain
LE392:pINT1	Hemoglobin-expressing strain [J. Mol. Biol. 210, 79-89, 1989]

The experimental cultures contained 12.5 mL of glucose semi-defined medium (see EXAMPLE 32) in 250 mL erlenmeyer flasks shaken at 250 rpm in a New Brunswick G24 incubator at 37°C. The flasks were seeded with a 0.5% (v/v) inoculum of late expoinential-phas cells. The culture optical

densities (ODs) were measured between 22-24 hrs, when the cultures had reached their maximum ODs. The maximum cell ODs are shown below:

Strain	Comment
LE392	6.06
LE392:pUC18	6.02
LE392:pGYM	9.70
LE392:pINT1	10.14

As can be seen, the myoglobin (LE392:pGYM) and the hemoglobin LE392:pINT1) cells outgrew the control cells by 60% and 67%, respectively. This result indicates that myoglobin provides a similar benefit to cell growth as Vitreoscilla hemoglobin. Because the function of both proteins is to reversibly bind oxygen, it is probable that oxygen binding is the primary proeprty of these proteins that reuslsts in growth improvement.

EXAMPLE 34 - EXPRESSION OF MYOGLOBIN IS NOT STRAIN SPECIFIC

To test whether expression of myoglobin results in improved growth of an E. coli strain with a different genetic background than LE392, pGYM was transformed into strain MG1655 (K12, lambda-). A growth experiement similar to the previous example was performed. The only difference in the growth conditions ws that Tris-HCl was not added to the medium. This resulted in slightly lower maximum ODs. The pGYM (myoglobin) strain was compared to the pUC18 control strain. The maximum culture ODs are shown below.

Strain	Maximum OD (A600)
MG1655:pUC18	5.23
MG1655:pGYM	6.63

Thus, E. coli strain MG1655 expressing myoglobin demonstrates a 27% improvement in growth over the control, indicating that the beneficial effect of myoglobin is not strain specific.

EXAMPLE 36 - GROWTH OF ENHANCEMENT OF *E. COLI* IN BATCH FERMENTATION

The growth properties are compared of the recombinant strains expressing Vitreoscilla hemoglobin (LE392:pINT1), horse heart myoglobin (LE392:pGYM), soybean leg hemoglobin (LE392:pKK-LHbc1), and E. coli hemoprotein (LE392:pTac-HMP) with similr plasmid-containing (LE392:pUC18) and plasmid free (LE392) cells under typical batch fermentation conditions.

Cells were grown in B. Braun MD fermentors at 37±0.5°C and pH of 7±0.05 with a working volume of 2 L in the semi-defined medium described in Example C. A constant air flow rate of 2 L/min and agitator speed of 700 rpm were maintained throughout each run. In all cases, the dissolved oxygen (DO) levels remained above 5% of air saturation under those aeration conditions.

Results:

The growth parameters measured for the six strains are listed in the following table:

	LE392: pUC18	LE392: pINT1	LE392: pGYM	LE392: pKK-LHbc1	LE392: pTac-HMP	LE392 (PLASMID FREE)
Log-phase growth rate (1/h)	0.76	0.95	0.95	1.03	0.87	0.92
Stationary phase cell density OD (600 nm)	4.2	7.6	7.5	7.6	7.4	7.5
Final total protein (g/L)	0.96	1.15	1.22	1.30	1.36	1.28

Conclusion:

Cells containing hemoglobins grow faster and to higher cell densities than comparable plasmid-containing contr ls.

EXAMPLE 36 - THE PRESENCE OF OXYGEN BINDING HEMOGLOBINS IN CORYNEBACTERIUM GLUTAMICUM ENHANCES L-LYSINE PRODUCTION AND YIELD.

In this example, production of lysine in batch termination by C. glutamicum ATCC 13287 transformed with plasmid pFS1 (Vitreoscilla hemoglobin) and with plasmid pMYO (myoglobin) was measured and compared to that of the parent strain (no plasmid).

C. glutamicum ATCC 13287:pFS1, C. glutamicum ATCC 13287:pMYO, and C. glutamicum ATCC 13287:no-plasmid cells were grown in 250-mL shake flasks at 30°C and 250 rpm in the following synthetic medium: glucose, 60 g/L; yeast extract, 2 g/L; ammonium sulfate, 55 g/L; magnesium sulfate (heptahydrate), 0.8 g/L; potassium phosphate, 1 g/L; manganese sulfate (tetrahydrate), 0.01 g/L; ferrous sulfate (heptahydrate), 0.01 g/L; biotin, 100 µg/L; thiamine-HCl, 200 µg/L; L-leucine, L-methionine and L-threonine, 200 mg/L each; pH 7.0. These cells were used to seed the fermentors.

Fermentations were conducted in 3-L B. Braun MD fermentors in the medium described above, at 30°C and udner constant air sparging (1.0 L/min). Initially, the impeller agitation rate was constant. During that time, the dissolved oxygen concentration gradually decreased. When the dissolved oxygen concentration reached 5% of air saturation, the agitation rate was adjusted by the fermentor controller in order to maintain the dissolved oxygen concentration at 5% of air saturation until the end of the fermentations. The culture pH was maintained

at pH 7.0 by the ferrrmentor pH controller by periodic additions of 4 N sodium hydroxide.

Samples were taken at different times through ut the fermentations. Glucose and lysine concentrations were determined by HPLC. Optical density was measured at 600 nm using a Beckman spectrometer. An OD_{600} of 1.0 corresponds to a cell mass of 0.35 g dw/L. Vitreoscilla hemoglobin and myoglobin expression was stable throughout the fermentation as demonstrated by Western electroblotting of the samples taken. The oxygen-binding proteins produced by both 13287.pFS1 and the myoglobin produced by 13287:pMYO were biologically active as demonstrated by carbon monoxide binding assay.

Table 2.1 shows the optical density, glucose and lysine concentration as a function of time for 13287:pFS1, 13287:pMYO, and 13287:np.

Table 2.1:

Time (h)		OD_{600}	glucose (g/L)	lysine (g/L)
7.5	13287:pFS1	16.3	43.8	0.8
	13287:pMYO	17.0	37.2	1.0
	13287:np	23.4	38.5	1.5
24	13287:pFS1	45.2	7.7	8.2
	13287:pMYO	48.4	8.4	8.1
	13287:np	46.0	7.1	6.6
28	13287:pFS1	52.4	0.0	12.0
	13287:pMYO	51.6	0.0	11.3
	13287:np	53.2	0.0	9.3

The cell yield per glucose consumed is similar in all the strains: 305 gcells/kgglucose for 13287:pFS1, 302 gcells/kgglucose for 13287:pMYO, and 310 gcells/kgglucose for 13287:np. The lysine yield per glucose consumed is 200 g/kkglucose for 13287:pFS1, 188 g/kgglucose for 13287:pMYO, and 155 g/kgglucose

for 13287:np. Vitreoscilla hemoglobin-containing cells have a lysine yield 29% higher than the no-plasmid cells, and myoglobin-containing cells have a lysine yield 21% higher than the no-plasmid cells. Lysine productivity per cell mass is 31% higher in the Vitreoscilla hemoglobin containing cells (23.4 g/kgcells/h for 13287:pFS1 and 17.9 g/kgcells/h for 13287:np). Also, lysine productivity per cell mass is 25% higher in the myoglobin containing cells (22.3 g/kgcells/h for 13287:pMYO and 17.9 g/kgcells/h for 13287:np).

These results indicate that expression of an active oxygen binding protein such as Vitresocilla hemoglobin or myoglobin in Coryneforms increases lysine accumulation; yield, and productivity.

SEQUENCE LISTIN

(1) GENERAL INFORMATION:

(i) APPLICANT:

Bailey, J. et al.

(ii) TITLE OF INVENTION:

ENHANCEMENT OF CELL GROWTH BY EXPRESSION AS A CLONED OXYGEN-BINDING PROTEINS

(iii) NUMBER OF SEQUENCES:

2

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(E) COUNTRY:

United States of America

(F) ZIP:

94104-2878

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE:

Diskette, 3.50 inch, 1.2 Kb storage

(B) COMPUTER:

IBM PC/XT/AT compatable

(C) OPERATING SYSTEM:

MS Dos 3.3

(D) SOFTWARE:

Word Perfect 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

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07/741,789

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07/151,256

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(B) FILING DATE:

05/20/92

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:

745 base pairs

(B) TYPE:

nucleic acid

(C) STRANDEDNESS:

single

(D) TOPOLOGY:

linear

(ii) MOLECULE TYPE:
cDNA to genomic RNA
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(v) ORIGINAL SOURCE:

(A) ORGANISM:
(B) STRAIN: Vitreoscilla sp.
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENT STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(vi) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE: pRED2

(ix) PUBLICATION INFORMATION:
(A) AUTHORS: Wakabayashi et. al.
(B) TITLE:
(C) JOURNAL: Nature
(D) VOLUME: 322
(E) ISSUE:
(F) PAGES 483
(G) DATE: 1986
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES:

(x) A SEQUENCE DESCRIPTION:SEQ ID NO:1:

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AAGCTTAACG  GACGCTGGGG  TTAAAAGTAT  TTGAGTTTTG  ATGTGGATTA  AGTTTTAAGA      60

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ATGAACTTAA  GGAAGACCCT  C ATG TTA GAC CAG CAA ACC ATT AAC ATC ATC AAA GCC ACT  180
                        Met Leu Asp Gln Gln Thr Ile Asn Ile Ile Lys Ala Thr

GTT CCT GTA TTG AAG GAG CAT GGC GTT ACC ATT ACC ACG ACT TTT TAT AAA AAC TTG TTT  240
Val Pro Val Leu Lys Glu His Gly Val Thr Ile Thr Thr Thr Phe Tyr Lys Asn Leu Phe

GCC AAA CAC CCT GAA GTA CGT CCT TTG TTT GAT ATG GGT CGC CAA GAA TCT TTG GAG CAG  300
Ala Lys His Pro Glu Val Arg Pro Leu Phe Asp Met Gly Arg Gln Glu Ser Leu Glu Gln

CCT AAG GCT TTG GCG ATG ACG GTA TTG GCG GCA GCG CAA AAC ATT GAA AAT TTG CCA GCT  360
Pro Lys Ala Leu Ala Met Thr Val Leu Ala Ala Ala Gln Asn Ile Glu Asn Leu Pro Ala

ATT TTG CCT GCG GTC AAA AAA ATT GCA GTC AAA CAT TGT CAA GCA GGC GTG GCA GCA GCG  420
Ile Leu Pro Ala Val Lys Lys Ile Ala Val Lys His Cys Gln Ala Gly Val Ala Ala Ala

CAT TAT CCG ATT GTC GGT CAA GAA TTG TTG GGT GCG ATT AAA GAA GTA TTG GGC GAT GCC  480
His Tyr Pro Ile Val Gly Gln Glu Leu Leu Gly Ala Ile Lys Glu Val Leu Gly Asp Ala

GCA ACC GAT GAC ATT TTG GAC GCG TGG GGC AAG GCT TAT GGC GTG ATT GCA GAT GTG TTT  540
Ala Thr Asp Asp Ile Leu Asp Ala Trp Gly Lys Ala Tyr Gly Val Ile Ala Asp Val Phe

ATA CAA GTG GAA GCA GAT TTG TAC GCT CAA GCG GTT AGA T AAAGTTTCAG  GCCGCTTTCA  600
Ile Gln Val Glu Ala Asp Leu Tyr Ala Gln Ala Val Glu

GGACATAAAA  AACGCACCAT  AAGGTGGTCT  TTTTACGTCT  GATATTTACA  CAGCAGCAGT      660

TTGGCTGTTG  GCCAAAACTT  GGGACAAATA  TTCGCCTGTG  TAAGAGCCCG  CCGTTGCTGC     720

GACGTCTTCA  GGTGTGCCTT  GGCAT                                              745
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(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:
146 amino acids

(B) TYPE:
amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY:
linear

(ii) MOLECULAR TITLE:
protein

(A) DESCRIPTION:
Vitreoscilla hemoglobin

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: INTERNAL FRAGMENT

(iv) ORIGINAL SOURCE:

(A) ORGANISM: Vitreoscilla sp.

(ix) FEATURE:

(A) NAME/KEY: Vitreoscilla hemoglobin protein

(B) LOCATION:

(C) IDENTIFICATION METHOD: Western analysis

(D) OTHER INFORMATION: binds oxygen

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Wakabayashi et. al.

(B) TITLE:

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(G) DATE: 1986

(xi) A SEQUENCE DESCRIPTION:SEQ ID NO:2:

```
                    5                   10
Met-Leu-Asp-Gln-Gln-Thr-Ile-Asn-Ile-Ile-
                   15                   20
Lys-Ala-Thr-Val-Pro-Val-Leu-Lys-Glu-His-
                   25                   30
Gly-Val-Thr-Ile-Thr-Thr-Thr-Phe-Tyr-Lys-
                   35                   40
Asn-Leu-Phe-Ala-Lys-His-Pro-Glu-Val-Arg-
                   45                   50
Pro-Leu-Phe-Asp-Met-Gly-Arg-Gln-Glu-Ser-
                   55                   60
Leu-Glu-Gln-Pro-Lys-Ala-Leu-Ala-Met-Thr-
                   65                   70
Val-Leu-Ala-Ala-Ala-Gln-Asn-Ile-Glu-Asn-
                   75                   80
Leu-Pro-Ala-Ile-Leu-Pro-Ala-Val-Lys-Lys-
                   85                   90
Ile-Ala-Val-Lys-His-Cys-Gln-Ala-Gly-Val-
                   95                  100
Ala-Ala-Ala-His-Tyr-Pro-Ile-Val-Gly-Gln-
                  105                  110
Glu-Leu-Leu-Gly-Ala-Ile-Lys-Glu-Val-Leu
                  115                  120
Gly-Asp-Ala-Ala-Thr-Asp-Asp-Ile-Leu-Asp-
                  125                  130
Ala-Trp-Gly-Lys-Ala-Tyr-Gly-Val-Ile-Ala-
                  135                  140
Asp-Val-Phe-Ile-Gln-Val-Glu-Ala-Asp-Leu-
                  145                  150
Tyr-Ala-Gln-Ala-Val-Glu
```

CLAIMS

WHAT IS CLAIMED IS:

1. A rec mbinant-DNA method for production of an oxygen-binding protein in a host cell grown in the presence of of oxygen, said host cell being chosen and derived from a selection of cells consisting of microorganisms and cells of multi-cellular organisms selected from the group consisting of animals, plants and insects comprising:

(a) preparing a portable DNA sequence capable of directing a host cell to produce a protein having oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cell capable of expressing said protein;

(c) growing the host cell under conditions appropriate for expression of said protein.

2. A method according to Claim 1 wherein said host cell is grown in culture.

3. A method according to Claim 1 further comprising the steps:

(d) in either order:

(i) harvesting said protein; and

(ii) permitting said protein to assume an active structure whereby it possesses oxygen-binding activity.

4. The method of Claim 1, wherein said portable DNA sequence is introduced directly and integrated into the chromosome of a host cultured cell.

5. The method f Claim 1, wherein said portable DNA sequence is introduced into said host cultured cell by the following steps:

(a) cloning the p rtable DNA sequence into a vector capable of being transferred into and replicating in a host cell, such vector containing operational elements for the portable DNA sequence;

(b) transferring the vector containing the portable DNA sequence and operational elements into a host cultured cell capable of expressing said oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for replication and propagation of the vector and expression of said protein.

6. The method of Claim 1 wherein said host microorganisms are selected from the group consisting of bacteria, fungi, molds, and yeast.

7. The method of Claim 6 wherein said host organism comprises yeast.

8. The method of Claim 1 wherein said protein is harvested prior to being permitted to assume said active structure.

9. The method of Claim 1, wherein said protein is allowed to assume said active structure prior to being harvested.

10. The method of Claim 2, wherein said vector is amplified in a microbial host prior to transfer into the host cell.

11. A method according to Claim 5 wherein said host comprises Penicillium chrysogenum.

12. A process for subjecting the expression of a selected DNA sequence encoding an oxygen-binding protein to external control under given environmental conditions which comprises the steps of:

(a) providing at least one selected isolated structural gene that is responsive to a Vitreoscilla hemoglobin promoter/regulator DNA sequence under the given environmental conditions; and

(b) operatively fusing the selected structural gene with said promototer/regulator DNA sequence.

13. The process of Claim 12, wherein said structural gene is transcriptionally responsive.

14. The process of Claim 12, wherein said structural gene is translationally responsive.

15. A method for expressing an oxygen-binding protein in a host E. coli cultured cell, comprising:

(a) introducing into said host cultured cell a DNA expression vector containing a DNA sequence according to SEQUENCE No. 1, and DNA sequences coding for said oxygen-binding protein; and

(b) growing said host cultured cell in an appropriate medium and environment and isolating said oxygen-binding protein.

16. The method of Claim 15, wherein said DNA expression vector is a portable DNA sequence, and is introduced directly and integrated into the chromosome of a host cultured cell.

17. A method for increasing the growth characteristics, including the growth yield, the growth rate, and the achievable cell density under controlled circumstances, of a host cultured cell grown in the presence of oxygen, said host cultured cell being chosen and derived from a selection of cultured cells consisting of microorganisms and cells obtained from multi-cellular organisms selected from the group consisting of animals, plants and insects comprising:

(a) preparing a portable DNA sequence capable of directing said host cultured cell to produce a protein having at least some oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cultured cell capable of expressing at least some oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for expression of the protein.

18. The method of Claim 17, wherein said portable DNA sequence is introduced directly and integrated into the chromosome of a host cultured cell.

19. The method of Claim 17, wherein said portable DNA sequence is introduced into said host cultured cell by the foll wing steps:

(a) cloning the portable DNA sequence into a vector capable of being transferred into and replicating in a host cell, such vector containing operational elements for the portable DNA sequence;

(b) transferring the vector containing the portable DNA sequence and operational elements into a host cultured cell capable of expressing at least some of the oxygen-binding protein; and

(c) culturing the host cell under conditions appropriate for replication and propagation of the vector and expression of the protein.

20. A method for increasing the production of proteins, both those normally made and those expressed as a result of genetic engineering; biopolymers; amino acids; antibiotics; and other metabolic products, of a host cultured cell grown in the presence of oxygen, said host cultured cell being chosen and derived from a selection of cultured cells consisting of microorganisms and cells obtained from multi-cellular organisms selected from the group consisting of animals, plants and insects comprising:

(a) preparing a portable DNA sequence capable of directing a host cultured cell to produce a protein having oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cultured cell capable of expressing at least some oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for expression of the oxygen-binding protein.

21. A method according to Claim 20 wherein production of said oxygen-binding protein increases production of L-lysine in said host cell.

22. A method according to Claim wherein productions of said oxygen-binding protein increases production of Penicillin G in said host cell.

23. A method for transporting and supplying oxygen to oxygen-requiring processes and operations, comprising:

(a) preparing a portable DNA sequence capable of directing a host cultured cell to produce a protein having oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cultured cell capable of expressing at least some oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for expression of the protein;

(d) effectively delivering said host cell or a preparation from said host cell containing the oxygen-binding protein to said oxygen-requiring process.

24. A method for the binding and removal of oxygen from an environment comprising:

(a) preparing a portable DNA sequence capabl of directing a host cultured cell to produce a protein having oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cultured cell capable of expressing at least some oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for expression of the oxygen-binding protein; and

(d) effectively delivering said host cell or a preparation from said host cell containing the oxygen-binding protein to said oxygen-containing environment.

25. A method for increasing the growth characteristics, including the growth yield, the growth rate, and the achievable cell density under controlled circumstances, of a cell in culture, said host cultured cell being chosen and derivatized from a selection of cultured cells consisting of microorganisms and cells obtained from multicellular organisms selected from the group consisting of animals, plants, and insects, said cell being capable of producing an endogenous protein having at least some oxygen-binding activity, comprising culturing said host cell under conditions appropriate for expression of said oxygen-binding protein.

26. A method for increasing the production of proteins, both those normally made and those expressed as a result of genetic engineering,

biopolymers; amino acids; antibiotics; and other metabolic products, of a host cultured cell grown in the presence of oxygen, said host cultured cell being chosen and derived from a selection of cultured cells consisting of microorganisms and cells obtained from multi-cellular organisms selected from the group consisting of animals, plants and insects, said cell being capable of producing an endogenous a protein having at least some oxygen-binding activity, comprising culturing said host cell under conditions appropriate for expression of said oxygen-binding protein.

27. A method for the binding and removal of oxygen from an environment comprising the introduction of a host cultured cell to said oxygen-containing environment, said host cultured cell expressing a protein which possesses at least some oxygen-binding activity.

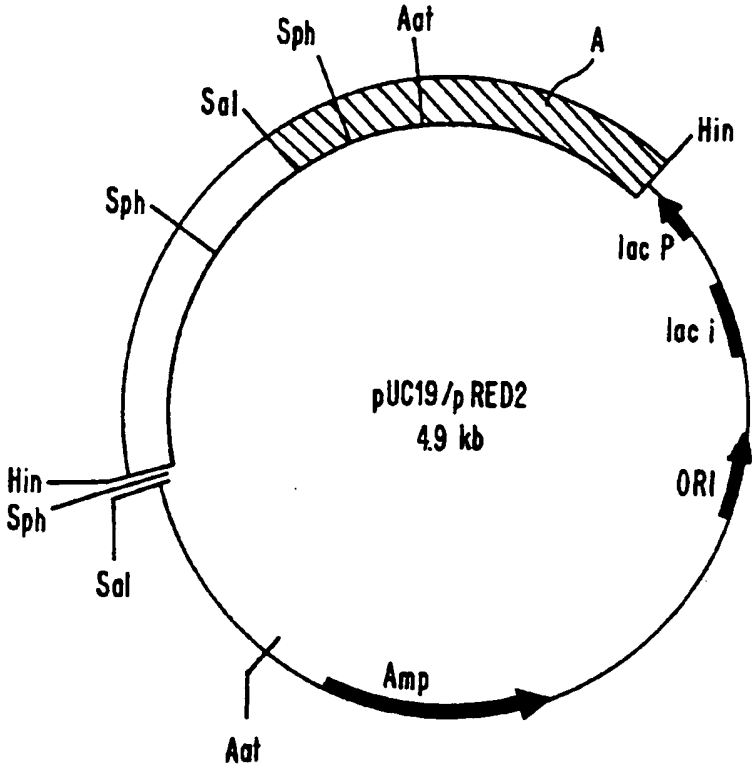
Sph
Aat
A
Sal
Hin
Sph
lac P
lac i
pUC19/p RED2
4.9 kb
ORI
Hin
Sph
Sal
Amp
Aat

FIG. 1.

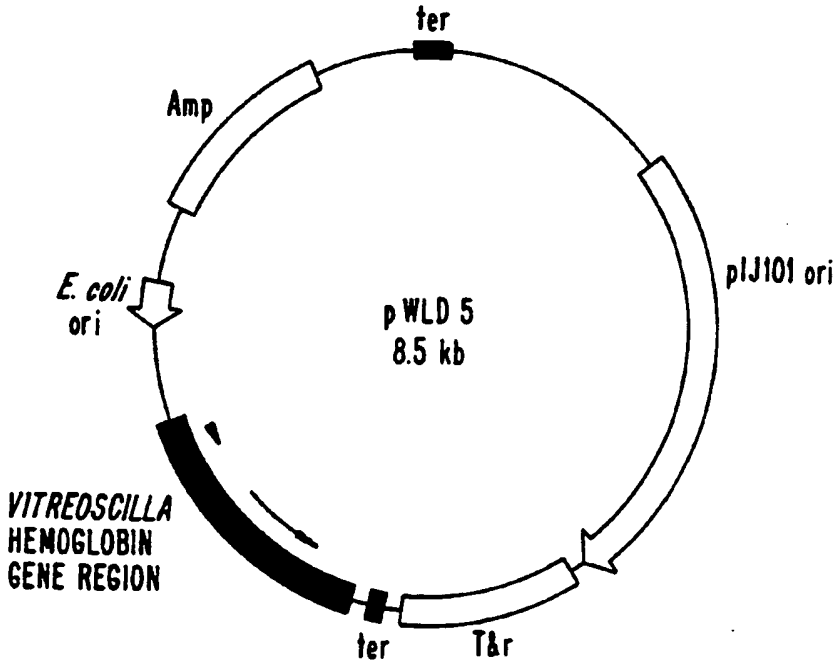
ter
Amp
pIJ101 ori
E. coli
ori
p WLD 5
8.5 kb
VITREOSCILLA
HEMOGLOBIN
GENE REGION
ter
T&r

FIG. 2A.

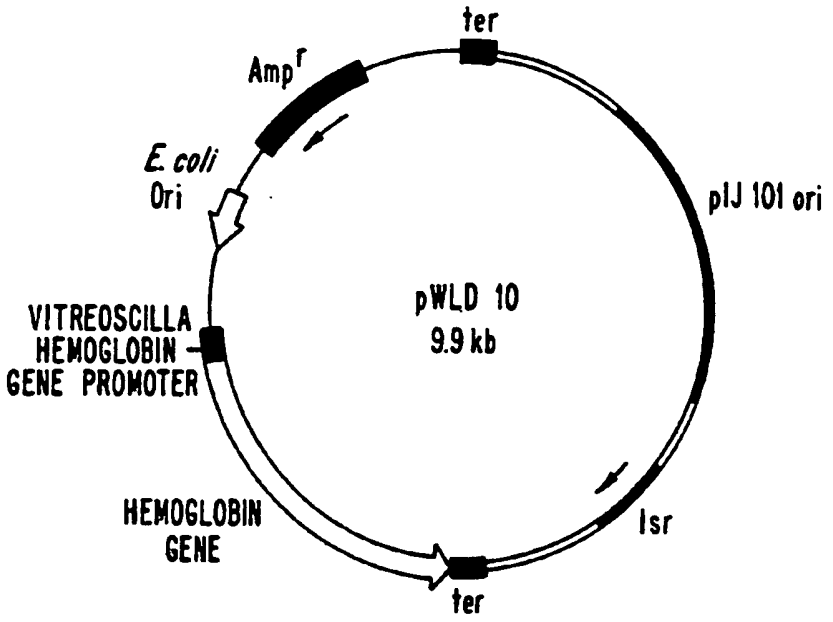
ter
Amp^r
E. coli
Ori
pIJ 101 ori
pWLD 10
9.9 kb
VITREOSCILLA
HEMOGLOBIN
GENE PROMOTER
HEMOGLOBIN
GENE
Isr
ter

FIG. 2B.

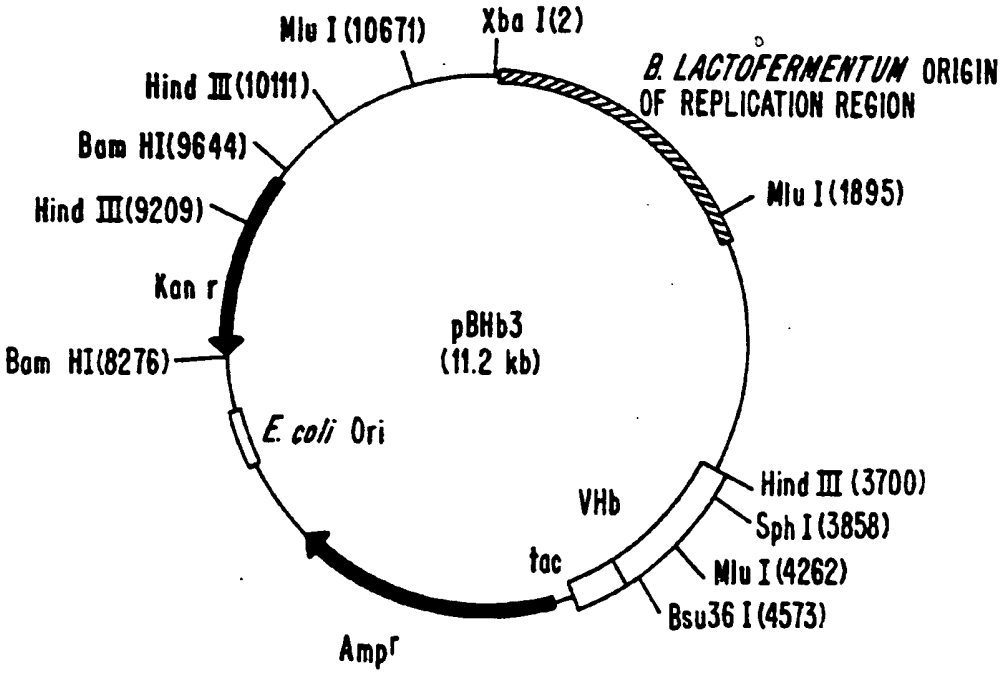
Mlu I (10671)
Xba I(2)
B. LACTOFERMENTUM ORIGIN
OF REPLICATION REGION
Hind III(10111)
Bam HI(9644)
Mlu I(1895)
Hind III(9209)
Kan r
pBHb3
(11.2 kb)
Bam HI(8276)
E. coli Ori
Hind III (3700)
VHb
Sph I (3858)
tac
Mlu I (4262)
Bsu36 I (4573)
Amp^r

FIG. 3.

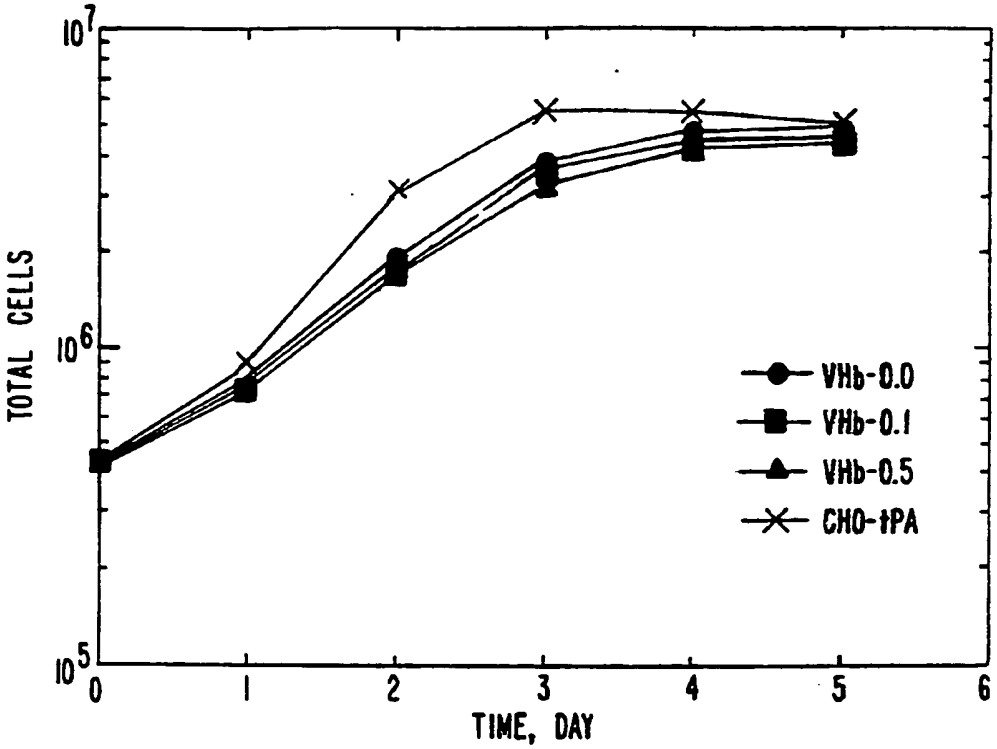
TOTAL CELLS
10^7
10^6
10^5
0
1
2
3
4
5
6
TIME, DAY
VHb-0.0
VHb-0.1
VHb-0.5
CHO-tPA

FIG. 4A.

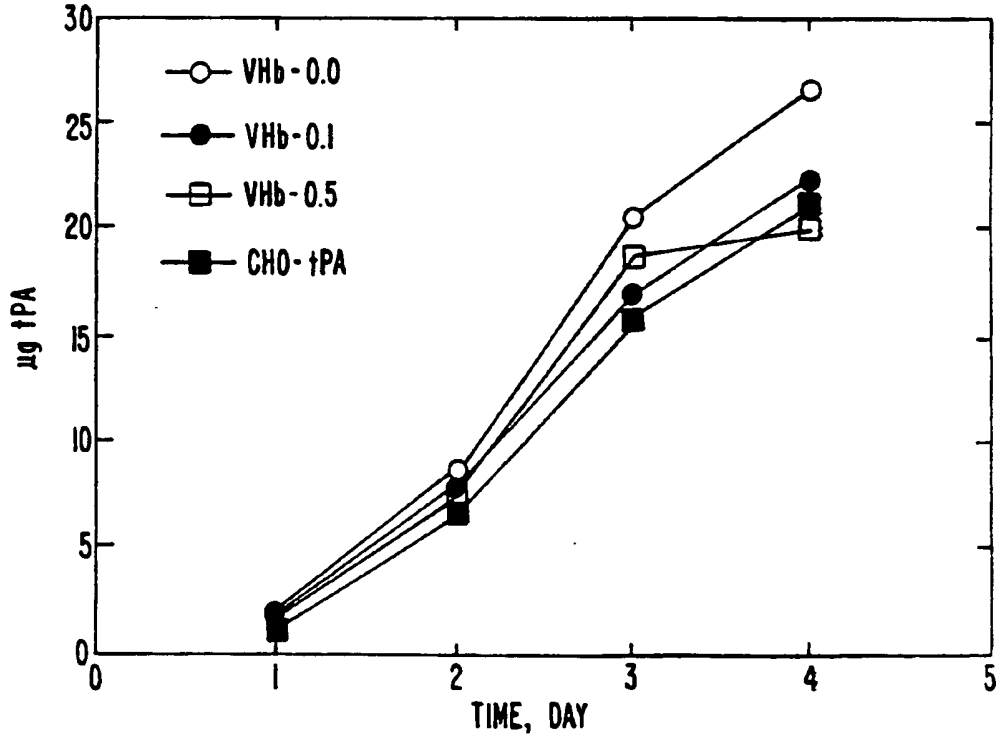
µg tPA
30
25
20
15
10
5
0
0
1
2
3
4
5
TIME, DAY
VHb-0.0
VHb-0.1
VHb-0.5
CHO-tPA

FIG. 4B.

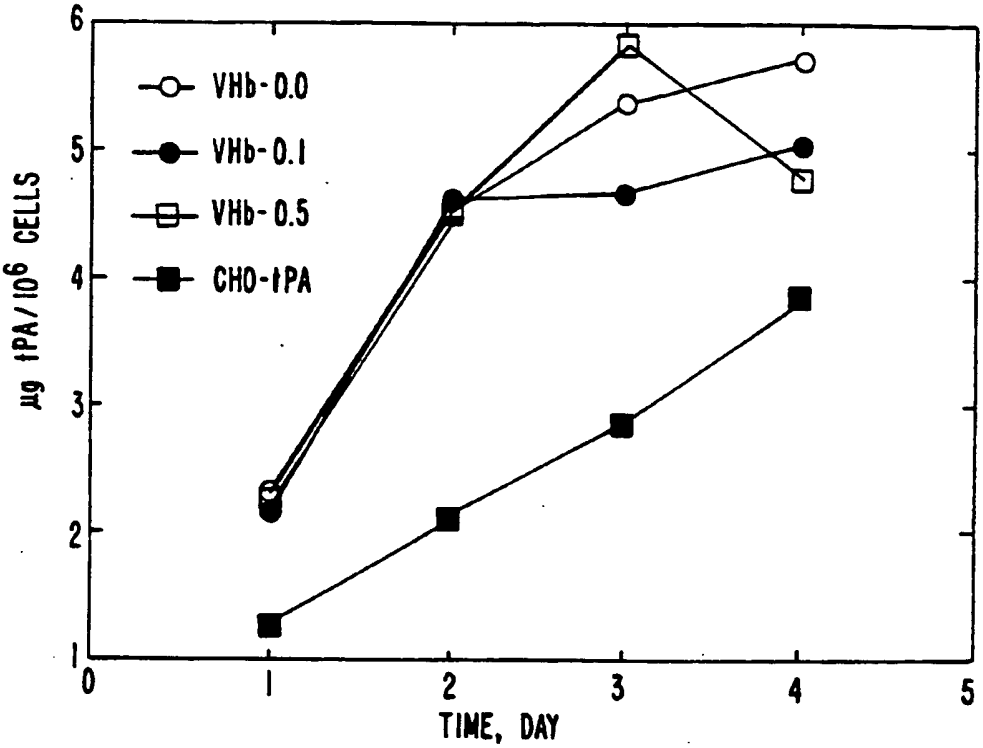
VHb-0.0
VHb-0.1
VHb-0.5
CHO-tPA
µg tPA/10^6 CELLS
TIME, DAY
0
1
2
3
4
5
6

FIG. 4C.

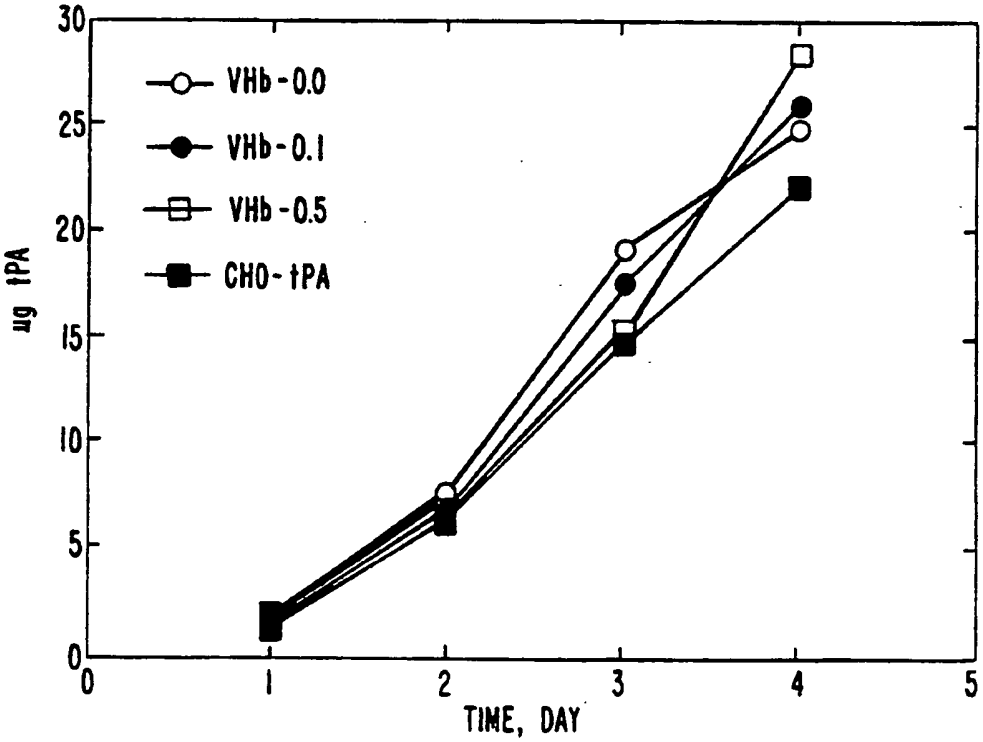
VHb-0.0
VHb-0.1
VHb-0.5
CHO-tPA
µg tPA
TIME, DAY
0
5
10
15
20
25
30
1
2
3
4
5

FIG. 5A.

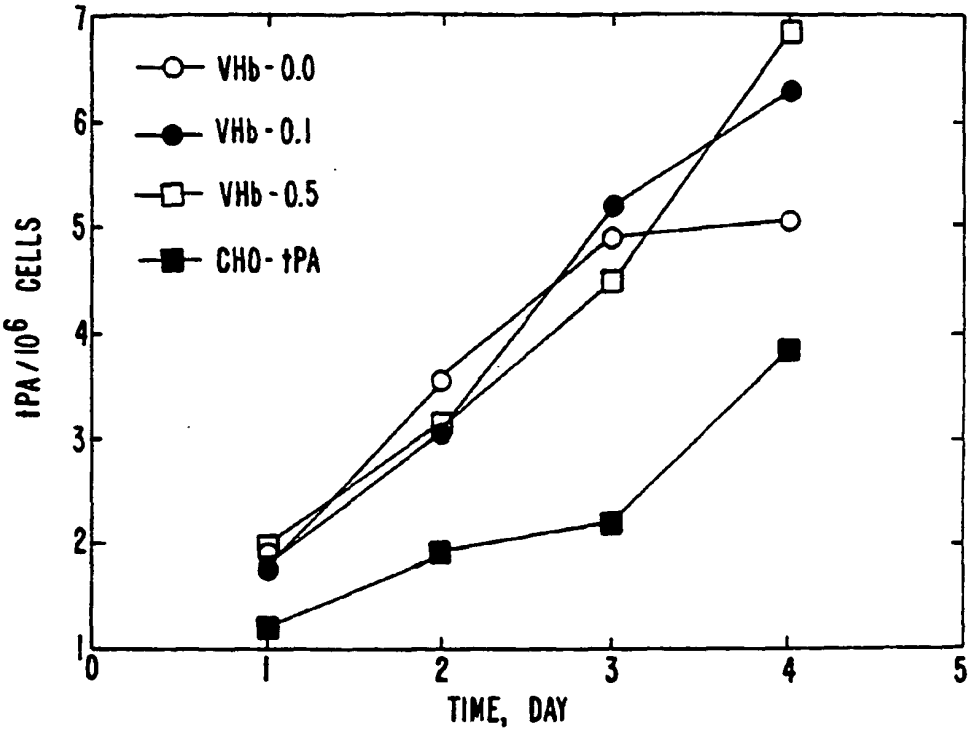
VHb - 0.0
VHb - 0.1
VHb - 0.5
CHO - tPA
tPA/10^6 CELLS
TIME, DAY
0
1
2
3
4
5
6
7

FIG. 5B.

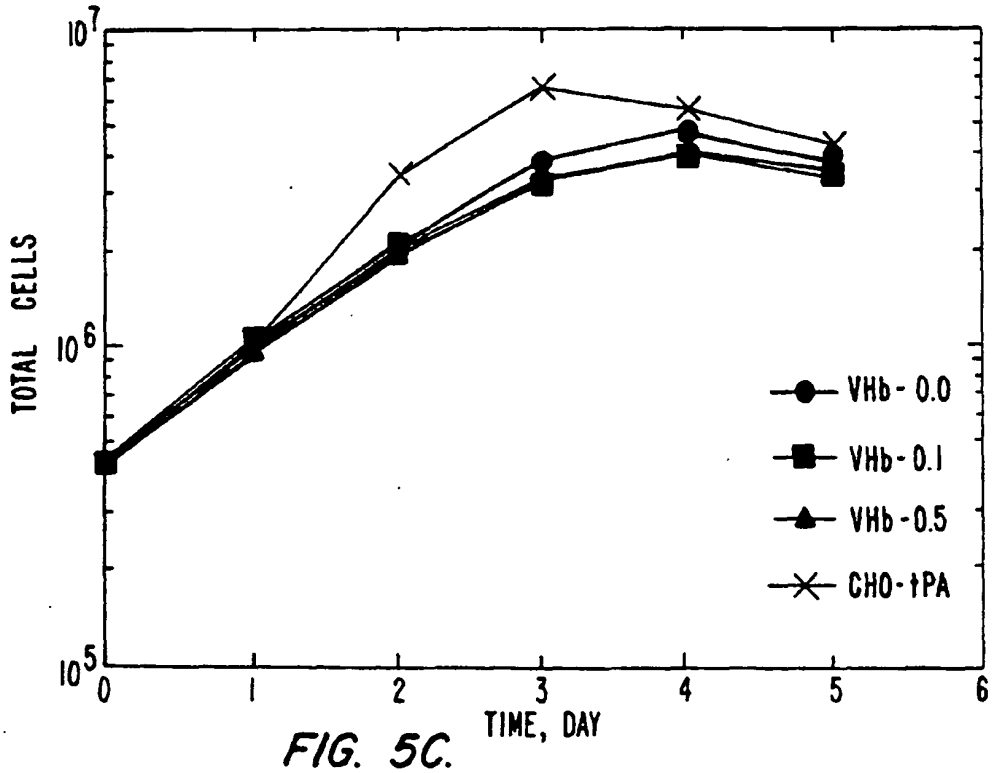
VHb - 0.0
VHb - 0.1
VHb - 0.5
CHO - tPA
TOTAL CELLS
TIME, DAY
10^5
10^6
10^7
0
1
2
3
4
5
6

FIG. 5C.

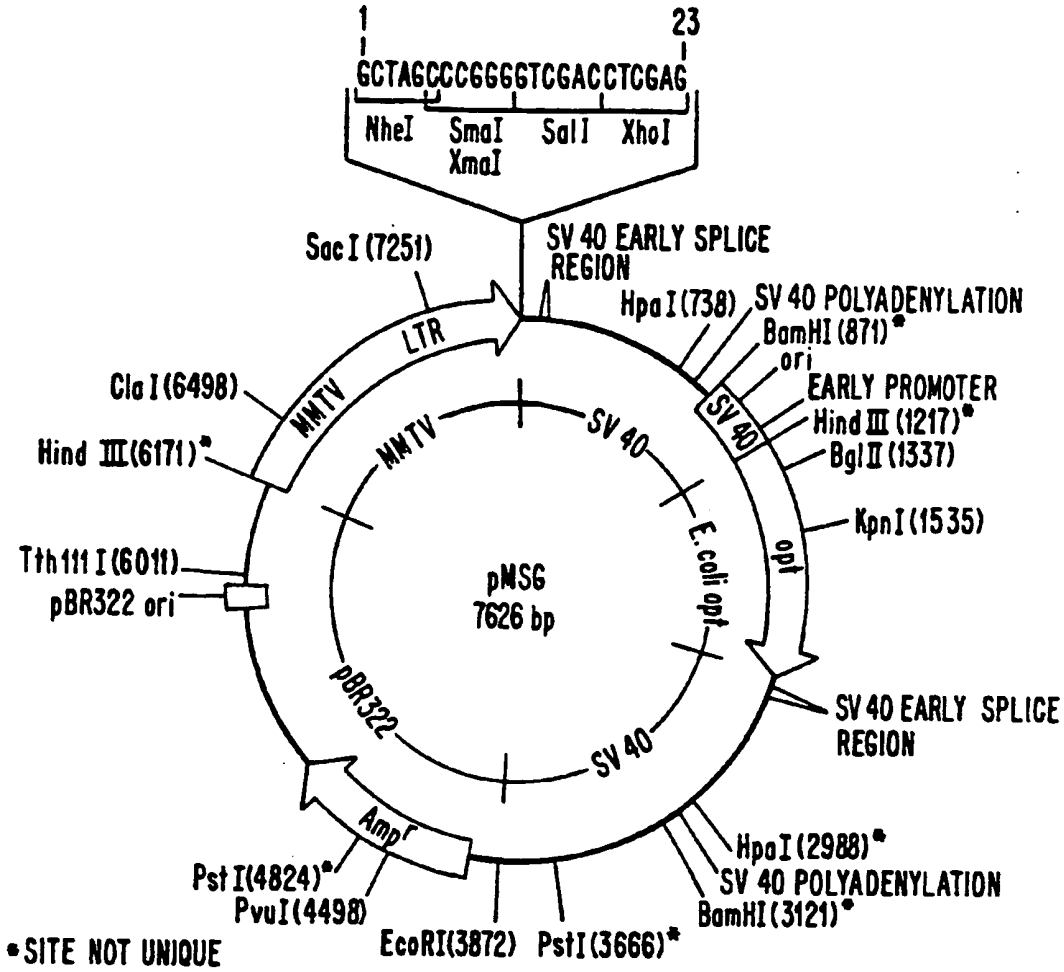
1
23
GCTAGCCCGGGGTCGACCTCGAG
NheI
SmaI
XmaI
SalI
XhoI
SacI (7251)
SV 40 EARLY SPLICE REGION
HpaI (738)
SV 40 POLYADENYLATION
BamHI (871)*
ori
EARLY PROMOTER
HindIII (1217)*
BglII (1337)
KpnI (1535)
opt
SV 40 EARLY SPLICE REGION
HpaI (2988)*
SV 40 POLYADENYLATION
BamHI (3121)*
PstI (3666)*
EcoRI (3872)
PstI (4824)*
PvuI (4498)
AmpR
pBR322
SV 40
E. coli opt
SV 40
MMTV
LTR
MMTV
ClaI (6498)
HindIII (6171)*
Tth111I (6011)
pBR322 ori
pMSG
7626 bp
*SITE NOT UNIQUE

FIG. 6.

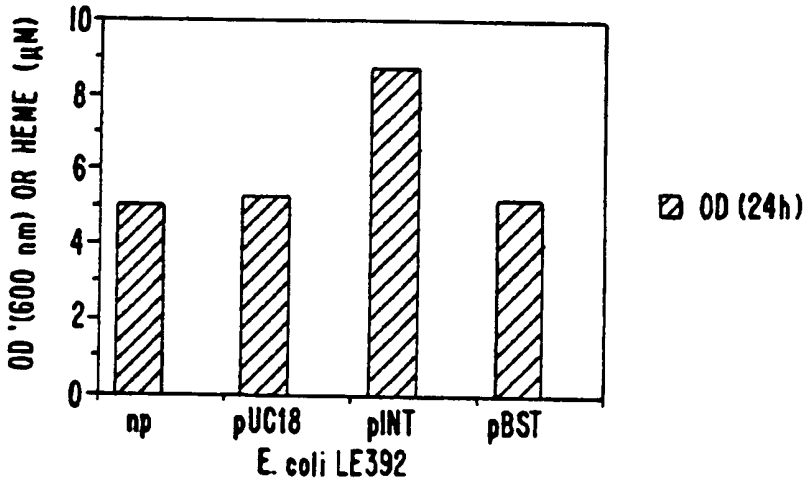
OD '(600 nm) OR HEME (μM)
10
8
6
4
2
0
np
pUC18
pINT
pBST
E. coli LE392
OD (24h)

FIG. 7.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05527

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12P 21/00, 1/00, 13/04, 13/08; 37/00; C12N 1/00, 15/00
US CL :435/69.1, 172.3, 41, 43, 106, 115, 317.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.3, 41, 43, 106, 115, 317.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

U.S. Automated Patent System, Biosis, Derwent Biotechnology Abstracts. Keywords: Vitreoscilla, hemoglobin, haemoglobin, oxygen, penicillin, chrysogenum, recombinant, vector, expression.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 89/03883 (KHOSLA ET AL) 05 MAY 1989, see claims 122, 37-39, 50-58.	1-10, 12-20, 23-27
X	WO, A, 92/03546 (SANDER ET AL) 05 MARCH 1992, see claims 8-12, 14-17	1, 2, 6, 20, 21, 26
X Y	WO, A, 91/06628 (HUGHES ET AL) 16 MAY 1991, see claims 6-8, 21-23, 27-29	1,2,6,17,20,25,26 11, 22
X Y	Bio/Technology, vol. 9, issued May 1991, Magnolo et al, "Actinorhodin production by Streptomyces coelicolor and growth of Streptomyces lividans are improved by the expression of a bacterial hemoglobin", pages 473-476, see entire document.	1,2,5,6,10,17,19, 20,25,26 4, 11, 18, 22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 JULY 1993	12 AUG 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer MARY E. MOSHER, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biotechnology and Bioengineering, vol. 22, no. 2, issued February 1980, Ryu et al, "Quantitative Physiology of Penicillium chrysogenum in Penicillin Fermentation", pages 289-298, see page 293, lines 1-12.	11, 22
Y	Biotechnology and Bioengineering, vol. 36, issued 20 December 1990, Ho et al, "Enhancing Penicillin Fermentations by Increased Oxygen Solubility Through the Addition of n-Hexadecane", pages 1110-1118, see abstract.	11, 22
Y	EP, A, 0,260,762 (MARTIN ET AL) 23 MARCH 1988, see entire document.	4, 11, 18, 22